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# Phytopathology

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## The American Phytopathological Society

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PHYTOPATHOLOGY

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# STUDIES ON A LETHAL PRINCIPLE EFFECTIVE IN THE PARASITIC ACTION OF TRICHODERMA LIGNORUM ON RHIZOCTONIA SOLANI AND OTHER SOIL FUNGI[1,2]

R. WEINDLING[3]

(Accepted for publication January 8, 1934)

## INTRODUCTION AND OUTLINE

In earlier papers (39, 40) the parasitism of *Trichoderma lignorum* (Tode) Harz on *Rhizoctonia solani* Kühn and on other soil organisms has been described. An investigation of the nature of this peculiar action has been started, using the same pigmented strain of *Trichoderma lignorum* and the strain of *Rhizoctonia solani* isolated from Citrus seedlings.

The principal object of this paper is to present evidence for the following fundamental aspects of the action:

1. The parasitic activity of the Trichoderma is made possible by a deadly substance, a lethal principle, which is excreted into the surrounding medium by the young hyphae, in presence or absence of other organisms. The time of the highest production of the lethal principle in absence of Rhizoctonia is in agreement with the period of greatest parasitic activity during the life cycle of the fungus when grown with Rhizoctonia.

2. The lethal principle is very unstable, especially at low hydrogen-ion concentrations. Its effect decreases, therefore, with decreasing hydrogen-ion concentration.

3. The pH ranges of most rapid decomposition of the lethal principle and of its decreasing effect on Rhizoctonia agree with the ranges at which little or no parasitic activity is displayed by Trichoderma when grown in conjunction with Rhizoctonia.

Methods and experiments leading up to the present work have been described elsewhere (41). Parts of this report are included here.

[1] Paper No. 296, University of California, Graduate School of Tropical Agriculture and Citrus Experiment Station, Riverside, California.

[2] A portion of this research has been submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Plant Pathology in the Graduate Division of the University of California.

[3] The writer here gratefully acknowledges the continuous interest and advice of H. S. Fawcett under whose direction this work was done. Thanks are due L. J. Klotz for many helpful suggestions.

## THE LETHAL PRINCIPLE[4] AND ITS PRODUCTION

### Review of Literature

Most of the literature on toxic substances produced by plant-pathogenic fungi is concerned with the wilts caused by different species of Fusarium. The usual procedure has been to grow the fungus in a rich nutrient medium, usually Richards' medium, for several weeks. The criterion for the toxic action of the filtrate obtained from these cultures is the wilting of cut plants or leaves placed into them. The following results have been obtained concerning the properties of these toxic principles of *Fusarium* spp. Most investigators find them heat-resistant (2, 10, 28, 31, 34, 42, 43). Some obtain colloidal (28, 42), others crystalloidal substances (17, 34, 42). There also is conflicting evidence as to partial volatility (20, 31, 34, 42, 43) and to the influence of pH (2, 10, 43). Usually, the toxic products lack the specificity displayed by the fungus (2, 4, 10, 23, 28, 43). In some cases, however, there is a similar reaction of susceptible and resistant varieties of the host plant to the pathogenic fungus and to its toxic principle (18, 31, 42).

Only two authors seem to have made an attempt to exclude the staling products of fungus cultures. Brown (5) tested the lethal principle of *Botrytis cinerea* by microscopic observation of its action on the cells of beet disks, while the macerating principle was examined macroscopically. The two principles seem to be inseparable. They have the character of an endoenzyme. Bewley (1), obtaining an "exoenzyme" from a *Verticillium* sp. causing "sleepy disease," finds its effect almost, but not entirely, destroyed by boiling. Brown (5) criticizes former work in which the killing effect of Botrytis has been ascribed to substances produced in culture media on which the fungus had been grown for some time. In these old solutions, staling substances are present that may not be produced by the young and actively growing fungus, and yet exert a certain toxic effect on the plant tissue. Perhaps this also will explain some of the contradictory results of workers with the Fusarium wilts that have been cited above. Brown and Bewley are apparently the only workers to attempt to determine the period of greatest parasitic activity after spore germination, and to produce the toxic principle by growing the fungus for this period only. It is realized that some cases of parasitic action are too complex to allow the accurate determination of this period, but an approximate value may be obtained.

[4] The term "lethal principle" has been adopted because the toxic substance present in the filtrate of Trichoderma cultures actually kills the Rhizoctonia, while acetone extracts from mats of staling cultures of Trichoderma seem to have only an inhibiting effect. The macerating principle that causes the Rhizoctonia hyphae to split in later stages of the parasitic action has been found only in the filtrate of staling cultures of Trichoderma.

## Methods of Obtaining and Testing the Lethal Principle

Under the conditions of the present work, the period of greatest parasitic activity is 2 days after germination of the Trichoderma spores. This is shown by the fact that Rhizoctonia hyphae, when added to a freshly poured agar suspension of Trichoderma conidia, are most vigorously attacked at this time. In obtaining the lethal principle the Trichoderma is grown, therefore, for 2 days in 15 cc. of a liquid medium in Petri dishes. This method thus furnishes a concentrated filtrate from a fungus mat of rather uniform development.

An attempt has been made to develop uniform procedures of producing and testing the lethal principle. All the cultures were incubated at 24° C. All the operations were carried out as aseptically as possible. The Trichoderma was grown for 8 to 10 days on slants of glucose-potato agar. A heavy spore suspension was made from these slants and freed of hyphae and débris by filtration through a cone of No. 2 Whatman filter paper placed, prior to sterilizing, under the cotton plug inside a large test tube. About 14 million spores were added to each Petri dish containing 15 cc. of the following medium: 1,000 cc. water, 25 g. glucose, 2 g. bactopeptone, 2 g. $KH_2PO_4$, 1 g. $MgSO_4$, .01 g. $FeCl_3$. After 2 days' growth the resulting mycelial mats of Trichoderma were removed and the filtrate from a number of dishes was poured through a double layer of fine filter paper (No. 42). The composite sample was then divided into 10 cc. aliquot portions in test tubes. The few Trichoderma spores that occasionally escaped through the filter had no effect on the Rhizoctonia during the period of the experiments. The filtrate from cultures of 2 days' growth usually had the pH 4.5. The hydrogen-ion concentrations were determined by the quinhydrone electrode. The colorimetric method (6) was used for preliminary tests when quick work was imperative and also for pH values above 8.0.

Equivalent amounts of Rhizoctonia hyphae, approximately uniform in age and development, were obtained as follows: From the advancing margin of Petri dish cultures on glucose-potato agar, disks of 3 mm. diameter were cut and placed at equal distances in Petri dishes containing 10 cc. of water agar. The scant growth of Rhizoctonia formed within 3 days allowed good microscopical observation. From these cultures, disks 9 mm. in diameter were mounted on glass slides in paraffin cells used as a substitute for van Tieghem cells (41).

One-half cc. of the liquid to be tested was placed on each of 4 disks. The criterion of the lethal effect was the loss of the homogeneous appearance of the protoplasm and of the vacuolate structure of the Rhizoctonia hyphae. They appeared either empty or as if filled with a granular material (Fig. 1).

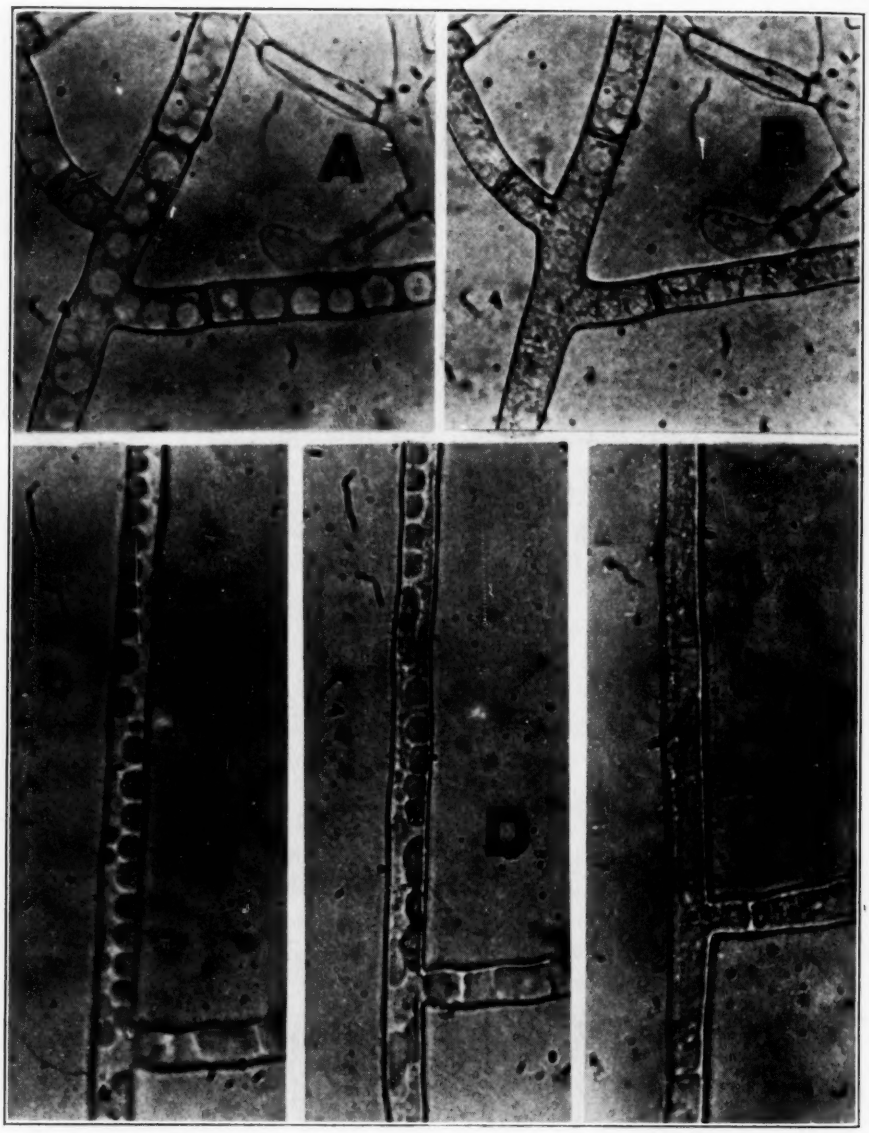


FIG. 1. Effect of the lethal principle on Rhizoctonia hyphae. A and C. Live hyphae, 5 minutes after mounting in undiluted filtrate of Trichoderma cultures. B and E. The same hyphae dead after one hour. D. Intermediate stage of hypha in C. C–E stained with neutral red. ×660.

The measure used here for evaluating the magnitude of the lethal effect of a given filtrate is called its lethal index. It is represented by the reciprocal of the lowest concentration at which all the hyphae of the Rhizoctonia are killed within 10 hours. Usually no further lethal effect is observed after this period. For example, if all the Rhizoctonia hyphae are killed within 10 hours by a maximum dilution of 1 in 40, the filtrate is considered to have the lethal index 40; if killed by a maximum dilution of 1 in 80, the lethal index is 80. If only a part of the Rhizoctonia hyphae is killed by the dilution 1 in 80, the lethal index is between 40 and 80, and is determined according to the percentage of Rhizoctonia hyphae killed. Under such an approximate system of computation, allowance for a considerable numerical variation must be made, especially at high dilutions. The amount of variation actually obtained seems to be not too excessive, in view of the difficulties in obtaining uniform procedures of production, test, and evaluation.

For the dilution series 1/10 molecular potassium phosphate buffers of the desired pH were used. The dilutions of the filtrate that were not sufficiently concentrated to be toxic and the buffers served as checks.

## Production of the Lethal Principle

If the Trichoderma is started simultaneously in a large number of Petri dishes and the combined filtrate of 3 of these dishes is tested for its lethal index at frequent intervals, a curve similar to that shown in figure 2 will

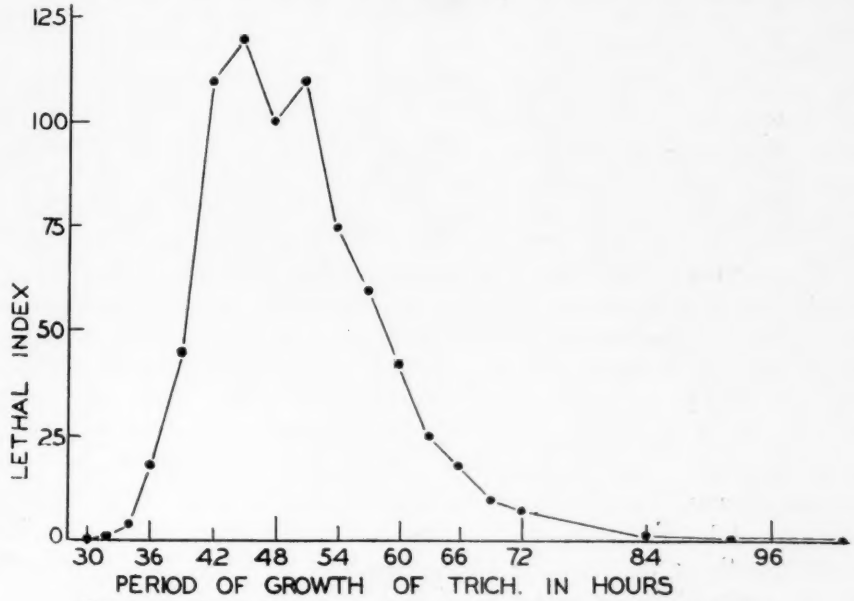


FIG. 2. Lethal indices obtained during the life cycle of Trichoderma.

be obtained. The data of 2 experiments of this type are given in table 1, the filtrate being tested at the original pH and at pH 2.5, to which it is adjusted.

TABLE 1.—*Lethal index of the combined filtrate of 3 Petri-dish cultures of Trichoderma lignorum at different periods of growth*

| Period of growth | Lethal index | | | |
|---|---|---|---|---|
| | Experiment 1 | | Experiment 2 | |
| | pH 4.5 | pH 2.5 | pH 4.5 | pH 2.5 |
| *hours* | | | | |
| 30 | 0 | 0.5 | 0 | 0 |
| 32 | ..... | ..... | 1 | 2 |
| 33 | 7 | 10 | ..... | ..... |
| 34 | ..... | ..... | 4 | 8 |
| 36 | 25 | 38 | 18 | 30 |
| 39 | 40 | 60 | 40 | 75 |
| 42 | 75 | 140 | 110 | 180 |
| 45 | 100 | 160 | 120 | 220 |
| 48 | 90 | 150 | 100 | 170 |
| 51 | ..... | ..... | 110 | 200 |
| 54 | 50 | 80 | 75 | 130 |
| 57 | 35 | 70 | 60 | 120 |
| 60 | 28 | 65 | 42 | 75 |
| 63 | 18 | 35 | 25 | 37 |
| 66 | 7 | 17 | 18 | 28 |
| 69 | ..... | ..... | 10 | 15 |
| 72 | ..... | ..... | 8 | ..... |
| 75 | 3 | 6 | ..... | ..... |
| 80 | 1 | 2 | ..... | ..... |
| 84 | ..... | ..... | 1 | 2 |
| 92 | 0 | 1 | 0.5 | 1 |
| 104 | ..... | 1 | ..... | 1 |
| 132 | ..... | 1 | ..... | 1 |

Figure 2 may be considered to give an approximate picture of the variation in quantities of the lethal principle produced at the different points of the life cycle of the fungus. There was a sudden rise in production during the second half of the second day, the maximum was near the end of this day, and after a rapid decrease during the third day the rate of production diminished slowly. Abundant sporulation occurred toward the end of this day. It should be emphasized also that during the third day of the growth of Trichoderma, the fungus mats will greatly increase in weight, indicating that autolytic processes have not yet set in. However, the rate of production of the lethal principle at this time has already greatly decreased. All attempts to obtain substances lethal to Rhizoctonia from the dried,

ground mycelial mats of Trichoderma have been unsuccessful. Thus, it appears that the formation of the lethal principle has no connection with autolytic processes. It is not impossible that the lethal principle is an intermediary product of metabolism that may be reutilized by the Trichoderma itself.

No lethal effect on the Rhizoctonia has been noted after more than 4 days when tested at pH 4.5. But, if the filtrate was adjusted to pH 2.5, lethal action was obtained up to the seventh day. Likewise, filtrates that become effective on Rhizoctonia only at pH values lower than 4.0, have been obtained from staling cultures of Trichoderma grown for several weeks on Richards' medium in Erlenmeyer flasks. This fact illustrates the inadequacy of this customary method for securing information on toxic substances if they are unstable. The period of maximum production under the conditions of these tests is around 48 hours after sowing the Trichoderma. At this time, which closely corresponds to the highest parasitic activity in the life cycle of the fungus, the lethal principle has been secured for use in the experiments described in the paragraphs that follow.

## DETERIORATION OF THE LETHAL PRINCIPLE AND THE RÔLE OF pH IN ITS EFFECTIVENESS

### Aerobic Deterioration

A characteristic property of the lethal principle is its rapid decomposition, which, under aerobic conditions, is retarded by low pH values and hastened by high pH values.

The data in table 2 have been obtained by adjusting 10 cc. aliquot portions of the filtrate to different pH values immediately after filtration, storing them at 24° C., and testing the lethal effect of these samples at frequent intervals. At pH 7.5 and 6.0, the lethal effect decreased within only $1\frac{1}{2}$ hours to about $\frac{1}{10}$ of the original value, and disappeared completely after 3 to 6 hours. At pH 4.5 and 2.5, however, periods 5 to 10 times longer were required to bring about the same changes.

During the time of testing the filtrates on the slides with Rhizoctonia, more of the lethal effect is lost at high pH values than at lower ones. The data in table 2 are, therefore, not directly comparable with one another. Another experiment has been conducted to make such a comparison possible. It is identical with those of table 2, except that all the portions of the filtrate stored at 3 different pH values are tested at one pH value, pH 4.5. The curves of figure 3 are thought to indicate the course of decomposition of the lethal principle during one day, as affected by the different pH values at which the decomposition takes place. These curves are in general agreement with the results of table 2, and are corroborated by the following experiments.

TABLE 2.—*Aerobic deterioration of the lethal principle of Trichoderma lignorum at 24° C. Lethal indices obtained at the pH of storing*

| Period of storage | Exp. 1 pH | | Exp. 2 pH | | Exp. 3 pH | | Exp. 4 pH | | | Exp. 5 pH | | | Exp. 6 pH | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4.5[a] | 2.5 | 4.5 | 2.5 | 4.5 | 2.5 | 6.0 | 4.5 | 2.5 | 7.5 | 6.0 | 4.5 | 7.5 | 6.0 | 4.5 |
| *hrs.* | | | | | | | | | | | | | | | |
| 0 | 90 | 140 | ..... | ..... | ..... | ..... | 45 | 130 | 240 | 25 | 60 | 120 | 19 | 37 | 110 |
| 1 | ..... | ..... | 110 | 200 | 65 | 100 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| 1.5 | ..... | ..... | ..... | ..... | ..... | ..... | 4 | ..... | ..... | 2 | 4 | 75 | 0.5 | 3 | 60 |
| 3 | 35 | 70 | 40 | 120 | 30 | 75 | 2 | 65 | 160 | 1 | 2 | 55 | 0 | 1.5 | 40 |
| 4.5 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 0 | 1 | 28 | ..... | 1 | 32 |
| 6 | 18 | 40 | 13 | 75 | 6 | 32 | 0 | 35 | 85 | ..... | 0 | 18 | ..... | 0.5 | 25 |
| 7.5 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 10 | ..... | 0 | 14 |
| 9 | 10 | 20 | 4 | 37 | 2 | 14 | ..... | 8 | 38 | ..... | ..... | ..... | ..... | ..... | ..... |
| 12 | 4 | 20 | 2 | 37 | 0.5 | 7 | ..... | 1 | 18 | ..... | ..... | ..... | ..... | ..... | ..... |
| 15 | 1 | 7 | ..... | ..... | ..... | ..... | ..... | 0 | 8 | ..... | ..... | ..... | ..... | ..... | ..... |
| 24 | 0 | 2 | 0.5 | 8 | 0 | 2 | ..... | ..... | 2 | ..... | ..... | ..... | ..... | ..... | ..... |
| 48 | ..... | ..... | 0 | 4 | ..... | 1 | ..... | ..... | 1 | ..... | ..... | ..... | ..... | ..... | ..... |
| 72 | ..... | ..... | ..... | 1 | ..... | 0.5 | ..... | ..... | 0.5 | ..... | ..... | ..... | ..... | ..... | ..... |
| 96 | ..... | ..... | ..... | 0 | ..... | 0 | ..... | ..... | 0 | ..... | ..... | ..... | ..... | ..... | ..... |

[a] The figures 7.5, 6.0, 4.5, and 2.5 of this line indicate the pH of storing and testing of the filtrate.

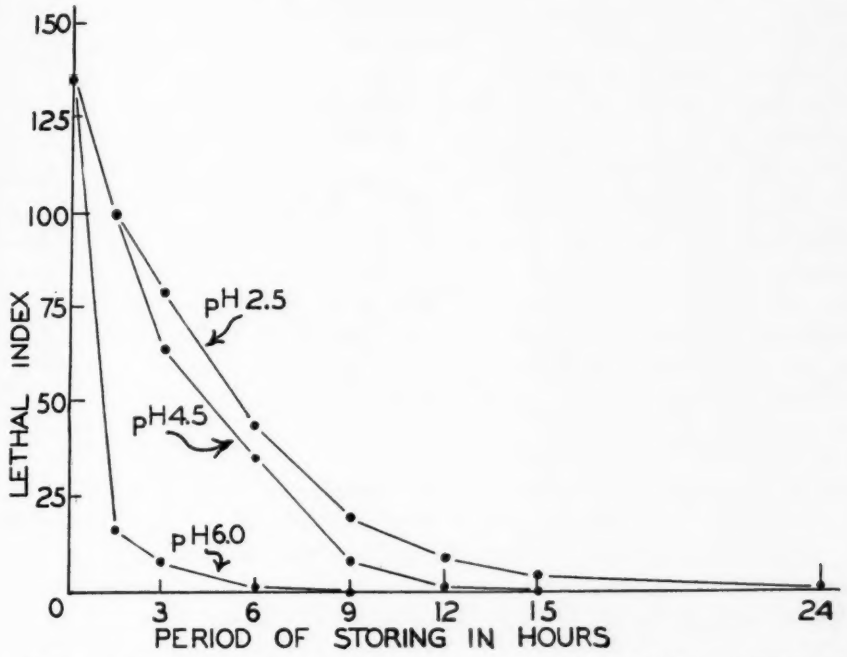


FIG. 3. Influence of pH on the deterioration of the lethal principle. Filtrates kept aerobically at 24° C.

The portions of a filtrate stored at different pH values show the greatest discrepancies as to their lethal indices after certain periods. Of these, 3 hours' storing has been chosen to compare filtrates kept at pH 6.0 and 4.5, and 12 hours' storing selected for contrasting samples stored at pH 4.5 and 2.5. Each aliquot portion was tested at the pH at which it had been stored,

TABLE 3.—*Aerobic deterioration of the lethal principle of Trichoderma lignorum at 24° C., as influenced by the pH of the filtrate*

| No. of experiment | Test pH | Lethal index | | | Test pH | Lethal index | | |
|---|---|---|---|---|---|---|---|---|
| | | Before storing | Stored 3 hrs. at | | | Before storing | Stored 12 hrs. at | |
| | | | pH 4.5 | pH 6.0 | | | pH 4.5 | pH 2.5 |
| 1 | 4.5 | 140 | 35 | 5 | | | | |
| | 6.0 | 38 | 9 | 1.5 | | | | |
| 2 | 4.5 | 75 | 30 | 4 | 4.5 | 75 | 1.5 | 10 |
| | 6.0 | 32 | 7 | 1 | 2.5 | 120 | 3 | 16 |
| 3 | 4.5 | 150 | 50 | 8 | 4.5 | 150 | 2.5 | 15 |
| | 6.0 | 60 | 14 | 2 | 2.5 | 240 | 4 | 28 |

as well as adjusted to the pH of the portion with which it was to be compared. The figures in table 3 indicate that at the chosen periods, the amounts of lethal principle were 5 to 7 times larger in the portions stored at the lower pH values as compared with those of the higher pH values.

There appears to exist a constant numerical relationship between the lethal indices obtained by adjusting one and the same filtrate to different pH values. In table 3 this relationship appears in the pairs of figures placed above one another. If tested at pH 6.0 the lethal index of any given sample is $\frac{1}{3}$ to $\frac{1}{4}$ of the lethal index obtained from the same sample at pH 4.5; if tested at pH 2.5, the lethal index is 1.5 to 2 times that of pH 4.5. The same relationship may be noted in tables 1, 5, and 6. This suggests that the chemical nature of the lethal principle is not influenced by the conditions under which decomposition takes place.

### Anaerobic *vs.* Aerobic Deterioration of the Lethal Principle

The decomposition of the lethal principle is retarded by low temperatures and by excluding the oxygen of the air. If stored 24 hours at 24° C. at pH 4.5 and tested at the same pH, the filtrate loses its lethal effect completely. In contrast, the lethal indices of filtrates stored at 2° C. or anaerobically at 24° C. will still amount to about one-third of the original

TABLE 4.—*Aerobic vs. anaerobic deterioration of the lethal principle of Trichoderma lignorum. Lethal index of filtrates stored and tested at pH 4.5*

| Period of storage | Experiment 1 | | Experiment 2 | | Experiment 3 |
|---|---|---|---|---|---|
| | Aerobic at 2° C. | Anaerobic at 24° C. | Aerobic at 2° C. | Anaerobic at 24° C. | Anaerobic at 24° C. |
| *days* | | | | | |
| 0 | 110 | 110 | 65 | 65 | 130 |
| ½ | 75 | 72 | 37 | 30 | ..... |
| 1 | 40 | 38 | 19 | 25 | ..... |
| 2 | 19 | 28 | 17 | 25 | 40 |
| 3 | 9 | 20 | 6 | 27 | ..... |
| 4 | 3 | 16 | ..... | ..... | ..... |
| 5 | 2 | 18 | 2 | 18 | ..... |
| 6 | 1 | 18 | ..... | ..... | ..... |
| 7 | 1 | 13 | 0.5 | ..... | ..... |
| 8 | 0.5 | 17 | ..... | ..... | ..... |
| 9 | ..... | ..... | 0 | ..... | ..... |
| 10 | 0 | ..... | ..... | 15 | ..... |
| 14 | ..... | 10 | ..... | ..... | ..... |
| 18 | ..... | ..... | ..... | ..... | 18 |
| 28 | ..... | 3 | ..... | ..... | ..... |
| 42 | ..... | ..... | ..... | 15 | ..... |
| 77 | ..... | ..... | ..... | ..... | 17 |

index (table 4). Samples in test tubes were kept anaerobically by placing them in tightly stoppered quart milk bottles from which the oxygen had been absorbed by the addition of 10 g. pyragallol and 100 cc. of 10 per cent KOH. While at 2° C., the lethal effect gradually disappeared within 10 days, it seemed to remain stationary for a long time under anaerobic conditions at a level represented by a lethal index of about 18 at pH 4.5. The longest period thus far recorded is 11 weeks. The apparent losses in one of the experiments presented in table 4 may be due to the repeated opening of the bottles.

Another feature of anaerobic decomposition is that it does not seem to be influenced by the pH of the filtrate if stored at 24° C. In table 5 each sample was tested as in table 3; that is, at the pH of storing as well as at the pH of the samples with which it was to be compared. The lethal indices of samples stored at different pH values, but tested at the same pH, were placed side by side. With anaerobic storing at 24° C., these pairs of lethal indices agree well within the limits of probable variations. With aerobic storing at 2° C., the lethal indices differ greatly according to the pH value at which the material is stored; *e.g.*, when stored at 2° C. at pH 4.5 for half a day, the amount of lethal principle retained is 20–30 times larger than when kept at pH 6.0 under the same conditions. The influence of pH on the anaerobic deterioration of the lethal principle at 2° C. cannot be explained completely with the data at hand. The differences in losses at dif-

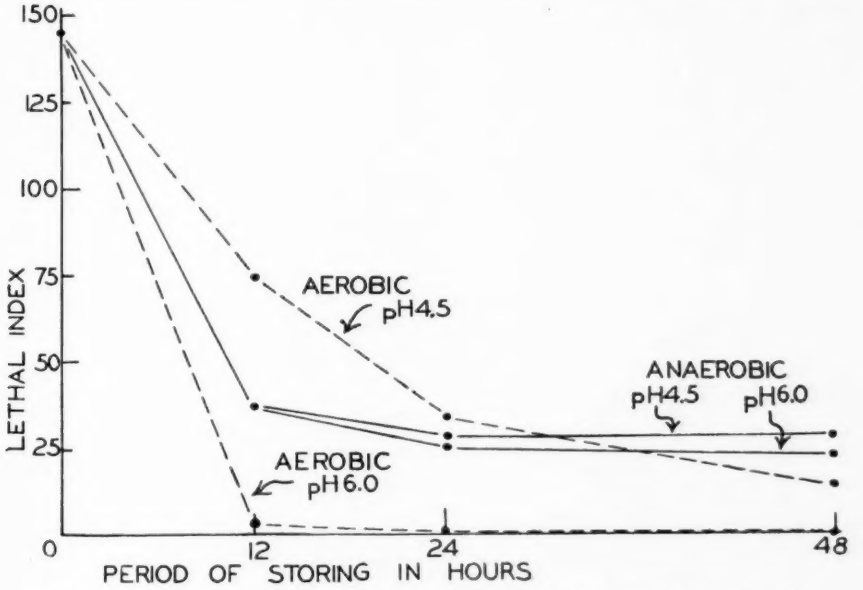


FIG. 4. Influence of pH on the deterioration of the lethal principle. Filtrates kept aerobically at 2° C. and anaerobically at 24° C.

TABLE 5.—*Aerobic and anaerobic deterioration of the lethal principle of Trichoderma lignorum, as influenced by the pH of the filtrate*

| Number of experiment | Period of storage | Test pH | Before storing | Lethal Index | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Aerobic storing | | | Anaerobic storing | | | | | |
| | | | | at 2° C. at pH | | | at 2° C. at pH | | | at 24° C. at pH | | |
| | | | | 6.0 | 4.5 | 2.5 | 6.0 | 4.5 | 2.5 | 6.0 | 4.5 | 2.5 |
| | *days* | | | | | | | | | | | |
| 1 | ½ | 6.0 | 60 | 1.0 | 20 | ..... | 10 | 25 | ..... | 17 | 15 | ..... |
| | | 4.5 | 145 | 4.0 | 75 | ..... | 27 | 85 | ..... | 38 | 40 | ..... |
| | 1 | 6.0 | ..... | 0.0 | 18 | ..... | 11 | 22 | ..... | 9 | 10 | ..... |
| | | 4.5 | ..... | 1.5 | 35 | ..... | 28 | 50 | ..... | 25 | 28 | ..... |
| | 2 | 6.0 | ..... | 0.0 | 5 | ..... | 9 | 18 | ..... | 10 | 10 | ..... |
| | | 4.5 | ..... | 0.0 | 15 | ..... | 32 | 40 | ..... | 23 | 30 | ..... |
| 2 | ½ | 6.0 | 40 | 0.5 | 18 | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| | | 4.5 | 100 | 2.0 | 65 | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| | 1 | 6.0 | ..... | 0.0 | 10 | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| | | 4.5 | ..... | 0.5 | 35 | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| | 2 | 6.0 | ..... | 0.0 | 4 | ..... | ..... | ..... | ..... | 5 | 5 | ..... |
| | | 4.5 | ..... | 0.0 | 19 | ..... | ..... | ..... | ..... | 18 | 20 | 15 |
| | | 2.5 | 140 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 30 | 22 |
| 3 | 1 | 4.5 | 50 | ..... | 4 | 18 | ..... | ..... | ..... | ..... | 7 | 6 |
| | | 2.5 | 80 | ..... | 10 | 25 | ..... | ..... | ..... | ..... | 18 | 18 |
| 4 | 1 | 4.5 | 80 | ..... | 25 | 50 | ..... | 25 | 45 | ..... | 12 | 10 |
| | | 2.5 | 150 | ..... | 65 | 120 | ..... | 75 | 110 | ..... | 35 | 30 |
| 5 | 1 | 4.5 | 60 | ..... | 8 | 22 | ..... | 20 | 25 | ..... | 12 | 10 |
| | | 2.5 | 100 | ..... | 20 | 45 | ..... | ..... | 42 | ..... | 30 | 25 |

ferent pH values are very much smaller, however, than with aerobic storing at 2° C. They tend to disappear with prolonged storing, thus approaching the effect of anaerobic storing at 24° C.

In figure 4, anaerobic and aerobic storing at pH 4.5 and 6.0 over a period of 2 days are contrasted as to their effect on the lethal action. With aerobic storing at 2° C., the relation of the two curves is similar to figure 3. The curves of anaerobic storing at 24° C., however, are almost identical, indicating that the chemical process of decomposition is different, according to whether it is effected with or without the oxygen of the air.

## Inactivation by Boiling

In many preliminary experiments the filtrate has been found to retain a small part of its lethal effect if boiled for one or more hours. Even 4 hours at 120° C. has not destroyed it completely.

The data of table 6 were obtained by adjusting aliquot amounts of the filtrate to different pH values and keeping them for 15 min. in a water

TABLE 6.—*Partial inactivation of the lethal principle of Trichoderma lignorum by boiling 15 min. at different pH values*

| Number of experiments | Test pH | Lethal index | | | |
|---|---|---|---|---|---|
| | | Before boiling | After boiling at pH | | |
| | | | 6.0 | 4.5 | 2.5 |
| 1 | 4.5 | 120 | 2.0 | 3.0 | 2.5 |
| 2 | 4.5 | 110 | 1.0 | 1.5 | 2.5 |
| 3 | 6.0 | 35 | 0.0 | 0.0 | ..... |
| | 4.5 | 100 | ..... | 2.0 | 3.0 |
| | 2.5 | 180 | ..... | 4.0 | 5.0 |

bath at 99° C. The decomposition of the lethal principle is very rapid, but seems to be rather independent of the pH at which the filtrate is boiled. It is possible that this is, chemically, a type of destruction similar to that occurring under anaerobic conditions. Since the lethal principle does not seem to be a mixture of substances, it may be concluded from this experiment that it is not of enzymatic nature.

## The Influence of pH on the Effectiveness of the Lethal Principle

Under normal environmental conditions the deterioration of the lethal principle proceeds faster the more alkaline the filtrate is made. Aliquot samples of filtrates were adjusted to a wide range of hydrogen-ion concentrations and their effect tested as simultaneously as possible (Table 7). The procedure involved delayed the testing of the whole series for at least $1\frac{1}{4}$ to $1\frac{1}{2}$ hours after filtration. After this time, no effect was obtained at

TABLE 7.—*The influence of pH on the effectiveness of the lethal principle of Trichoderma lignorum. Mounted 1–1½ hrs. after filtration and adjusting pH values*

| pH | Experiment 1 | Experiment 2 | Experiment 3 | Experiment 4 | Experiment 5[a] |
|---|---|---|---|---|---|
| 2.5 | 150 | ..... | 140 | 150 | 10 |
| 3.0 | ..... | ..... | 120 | 110 | 8 |
| 3.5 | 110 | ..... | 70 | 95 | ..... |
| 4.0 | ..... | ..... | 72 | 110 | 6 |
| 4.4 | ..... | ..... | 65 | 80 | 5 |
| 4.8 | 80 | 60 | 65 | 70 | ..... |
| 5.1 | ..... | 30 | 55 | 50 | 5 |
| 5.4 | ..... | 17 | 35 | 26 | ..... |
| 5.7 | ..... | 10 | 20 | 15 | 3 |
| 6.0 | 15 | 6 | 11 | 12 | 2 |
| 6.3 | ..... | 3 | 6 | 6 | ..... |
| 6.6 | ..... | 2 | 3 | 2 | ..... |
| 6.9 | ..... | 1 | 2 | 1 | 1 |
| 7.2 | 3 | 0.5 | 2 | 1 | ..... |
| 7.5 | ..... | 0 | 1 | 1 | 0 |
| 7.8 | ..... | ..... | 1 | 0.5 | ..... |
| 8.0 | 0 | ..... | 0.5 | 0 | 0 |
| 8.5 | ..... | ..... | 0 | 0 | ..... |
| 9.0 | ..... | ..... | 0 | 0 | ..... |
| 10.0 | ..... | ..... | 0 | 0 | ..... |

[a] Filtrate from Trichoderma cultures 3 days old.

pH 8.5. If this test was made, however, immediately after filtering and adjusting the pH, decided lethal effects were found up to pH 9.8. Nevertheless, the method permits one to visualize the effect of pH on the lethal principle over a wide range of pH values, though under an arbitrary set of conditions (Fig. 5). The curve showing the relationship of pH to lethal index decreases most rapidly between pH 4.8 and 6.3 after which it approaches the zero line more slowly.

## INTERACTION AND GROWTH OF TRICHODERMA LIGNORUM AND RHIZOCTONIA SOLANI, AS AFFECTED BY THE pH OF THE MEDIUM

### Methods

Methods for the study of the growth of fungi on liquid media maintained at fairly constant hydrogen-ion concentrations have been reported (24, 35). Since liquid media are not suitable for the microscopic observation of parasitism, most of the present work has been done with strongly buffered agar media of a low nutrient content and adjusted to a series

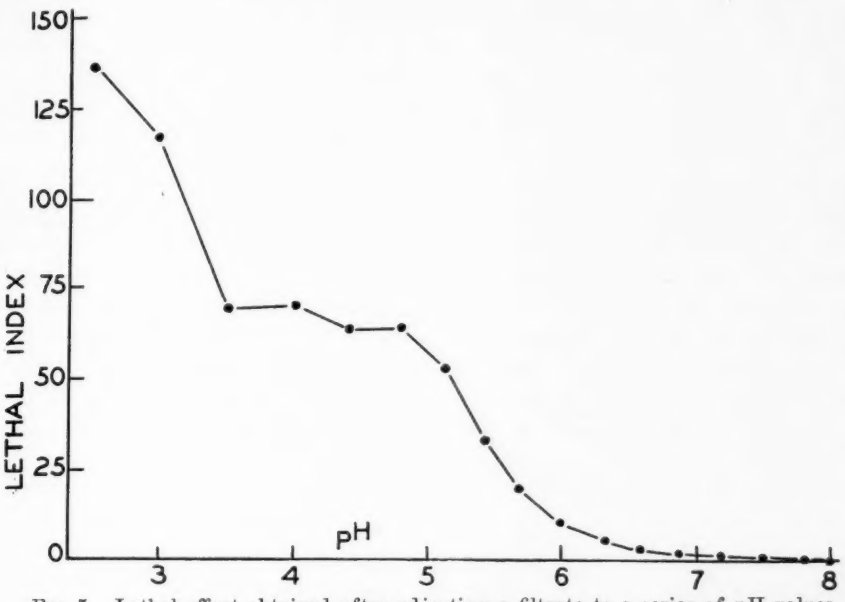


FIG. 5. Lethal effect obtained after adjusting a filtrate to a series of pH values.

of pH values having 0.5 pH intervals. The rather scant growth of the fungi does not tend to change the pH much, and allows good observation of their interaction.

All cultures of *Trichoderma lignorum* thus far isolated from the roots of plants, from decaying lemons, and from the soil have been found to attack the Rhizoctonia. They have been tentatively placed in 3 groups: The cultures of the *o* group have a characteristic cocoanut-like odor and readily cause decay of lemons when inoculated into wounds. The *p* and *q* groups possess neither of these properties. The *p* group differs from the other 2 groups by the formation of a yellow pigment under certain conditions. The *q* group appears to be distinguished from the *p* group only by the absence of the yellow pigment. Of the cultures used in this work $O_1$ and $O_2$ belong to the *o* group. $O_1$ is the stock culture of the Division of Plant Pathology at Riverside. $O_2$ is a recent isolation from lemons. The other cultures belong to the *p* group. $P_1$ has been used in previous work (40). $P_0$ and $P_2$ are single-spore cultures derived from $P_1$.

$P_0$ had lost a great deal of its parasitic activity, due apparently to repeated transfers of the vegetative mycelium. A single-spore culture of $P_3$, isolated by L. J. Klotz, from the gummosis lesion of an orange tree, has been used for the investigation of the lethal principle reported above.

In preliminary tests with $P_1$, Czapek's agar has been used, but with the nutrients and salts in one-half strength. All the other experiments

have been made with media strongly buffered with one-fifth molecular potassium phosphate. The buffer solution has been autoclaved separately and mixed with the tubes of agar just before pouring. In addition to the buffer salts, the media contain in 1,000 cc. water 20 g. agar, 2 g. glucose, 0.2 g. $MgSO_4$, a trace of $FeCl_3$, and 0.2 g. $KNO_3$. In the experiments with $O_2$ and $P_3$, 0.2 g. bacto peptone has served as a nitrogen source. The pH is tested in separate tubes by the quinhydrone electrode and, above pH 8.0, colorimetrically. Besides, 5 drops of previously sterilized indicator solutions are added to 1 of the 3 Petri dishes of each set in order to follow the changes of pH during growth. These indicators have not been found disturbing the growth of the fungi.

If only the influence of pH on the growth rates of fast-growing fungi is to be determined, weakly buffered media seem to give results similar to those of strongly buffered ones. The pH changes little in the advancing zone of the colonies, except at very high pH values. This would agree with the results of Fawcett (13) that the growth of certain fungi in solid media does not follow an autocatalytic curve, but continues unchanged because the tips of the advancing hyphae are always growing in media with the original pH. In the zone behind the advancing margin of the colonies, however, considerable changes of pH take place in weakly buffered media, tending toward a reaction between pH 6.0 and 7.0 in the work reported here. The buffered media maintain a nearly constant pH at acid and neutral reactions. The very alkaline media change about as follows: from an original pH 8.0 to 7.6, from pH 9.0 to 8.4, and from pH 10.0 to 8.8.

Most of the data of tables 8 and 9 are based on the average of 3 Petri dishes. The dishes were inoculated with disks of 3 mm. diameter from the advancing margin of fungus colonies on glucose-potato agar. In mixed cultures, the agar disks were placed 3 cm. from one another. The temperature of incubation was maintained at 24° C. in all experiments except that with nonbuffered media, where the temperature rose to 27° C. at times during the summer.

### Effect of pH on the Growth of Trichoderma and Rhizoctonia

The data of table 8 have been averaged from measurements in pure cultures that served as checks for the mixed-culture experiments. They also are thought to indicate the differences in relative chances of development of the 2 fungi caused by variations in the pH of soils. The influence of reaction upon the growth of many Rhizoctonia strains has been investigated by others (16, 25, 29, 32).

The Rhizoctonia grows well over the whole range of pH values occurring in natural soils. Even at pH 3.0, a thin hyphal web is formed. The optimum of the Citrus strain seems to extend from about pH 6.0 to pH 9.0

TABLE 8.—*The influence of pH on the growth of Rhizoctonia solani and Trichoderma lignorum. Diameters of colonies in mm. after 42 hrs. growth at 24° C.*

| pH | Trichoderma *p* group | | | | Trichoderma *o* group | | Rhizoctonia | | |
|---|---|---|---|---|---|---|---|---|---|
| | $P_1$[a] | $P_0$[b] | $P_2$[b] | $P_3$[c] | $O_1$[b] | $O_2$[c] | a | b | c |
| 1.7 | 0 | 0 | 0 | 6 | 0 | 7 | 0 | 0 | 0 |
| 2.0 | 8 | 10 | 8 | 7 | 15 | 23 | 0 | 0 | 0 |
| 2.5 | 22 | 24 | 28 | 38 | 28 | 39 | 0 | 0 | 7 |
| 3.0 | 45 | 31 | 43 | 57 | 35 | 49 | 25 | 27 | 34 |
| 3.5 | 61 | 46 | 49 | 65 | 37 | 56 | 58 | 48 | 52 |
| 4.0 | 70 | 53 | 54 | 65 | 40 | 60 | 67 | 58 | 55 |
| 4.5 | 73 | 49 | 57 | 63 | 43 | 60 | 71 | 60 | 55 |
| 5.0 | 72 | 40 | 60 | 61 | 41 | 60 | 78 | 62 | 55 |
| 5.5 | 70 | 42 | 57 | 61 | 42 | 56 | 79 | 65 | 57 |
| 6.0 | 69 | 43 | 54 | 57 | 42 | 54 | 82 | 67 | 61 |
| 6.5 | 66 | 37 | 47 | 43 | 37 | 38 | 84 | 72 | 64 |
| 7.0 | 64 | 33 | 38 | 37 | 29 | 25 | 85 | 76 | 64 |
| 7.5 | 51 | 28 | 27 | 27 | 22 | 15 | 88 | 79 | 66 |
| 8.0 | 44 | 14 | 13 | 14 | 12 | 5 | 89 | 72 | 65 |
| 8.5 | 41 | 13 | 10 | 13 | 10 | 5 | 90 | 70 | 65 |
| 9.0 | 32 | 11 | 9 | 11 | 0 | 4 | 92 | 69 | 63 |
| 9.5 | 20 | 0 | 0 | ..... | 0 | ..... | 90 | ..... | ..... |
| 10.0 | ..... | ..... | ..... | 0 | ..... | 2 | ..... | ..... | 63 |

[a] Nonbuffered modified Czapek's medium.
[b] Buffered nitrate medium.
[c] Buffered peptone medium.

and perhaps higher (Table 8). While the Trichoderma has a slight advantage at acid reactions, its growth rate decreases rapidly on the alkaline side of the range, the optimum range being about pH 4.0 to pH 6.0. This gives a great advantage to the Rhizoctonia in alkaline media (Fig. 6).

The difference seems to be large enough to rule out the possible objection that the effect of pH on the growth of the 2 fungi greatly influenced the nutritional and physical nature of the medium (25, 38).

There are slight differences between the growth rates of the Trichoderma cultures, especially the relatively better growth of the *o* group in very acid and of the *p* group in very alkaline media. However, the limited amount of data does not yet permit one to generalize these results. The good growth of Trichoderma in alkaline media of the nonbuffered series, as compared with the buffered ones, is due to a rapid change towards the neutral point in this first series.

### Effect of pH on the Parasitic Action

A summary of data concerning the parasitic action of Trichoderma on Rhizoctonia as influenced by the pH in a series of buffered media is presented in table 9.

TABLE 9.—*The influence of pH on the interaction of Trichoderma and Rhizoctonia*

| | Trichoderma cultures of the *p* group (pigmented) | | | | | | Trichoderma cultures of the *o* group (odor) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $P_0$[a] | | $P_2$ | | $P_3$ | | $O_1$ | | $O_2$ | |
| | Rhizoctonia hyphae | | | | | | Rhizoctonia hyphae | | | |
| pH | Dead[b] | Alive | Dead | Alive | Dead | Alive | Dead | Alive | Dead | Alive |
| 2.5 | | | | | All | None | | | All | None |
| 3.0 | All sf[c] | Most sm[c] | All | None | " | " | All | None | " | " |
| 3.5 | " " | " " | " | " | " | " | " | " | " | " |
| 4.0 | " " | " " | " | " | " | " | " | " | " | " |
| 4.5 | " " | " " | " | " | " | " | " | " | " | " |
| 5.0 | " " | All sm | " | " | " | " | " | " | " | " |
| 5.5 | ½ sf | " " | Most sf | ½ sm | All sf | Few sm | " | " | " | " |
| 6.0 | Few sf | " " | " " | Many sm | " " | Many sm | " | " | " | " |
| 6.5 | " " | " " | " " | " " | ½ sf | All sm | " | " | " | " |
| 7.0 | None | Some iv[c] | Few sf | Some iv | Few sf | Few iv | " | " | " | " |
| 7.5 | " | " " | " " | " " | " " | " " | Many sf | " | Most sf | — |
| 8.0[d] | " | " " | None | " " | None | " " | Some iv | Few iv | Some iv | — |
| 8.5 | " | " " | " | " " | " | " " | " " | " " | " " | — |
| 9.0 | " | " " | " | " " | " | " " | " " | " " | " " | — |

[a] *Trichoderma lignorum* cultures of *o* group and *p* group used for attack on Rhizoctonia.

[b] State of the Rhizoctonia hyphae in the attacked parts of the colonies 5–8 days after inoculation.

[c] Abbreviations concerning Rhizoctonia hyphae: sf = on the surface of the medium; sm = submerged in the medium; iv = invading the Trichoderma colony.

[d] Above pH 7.5 there is very little intermingling of Rhizoctonia and Trichoderma colonies.

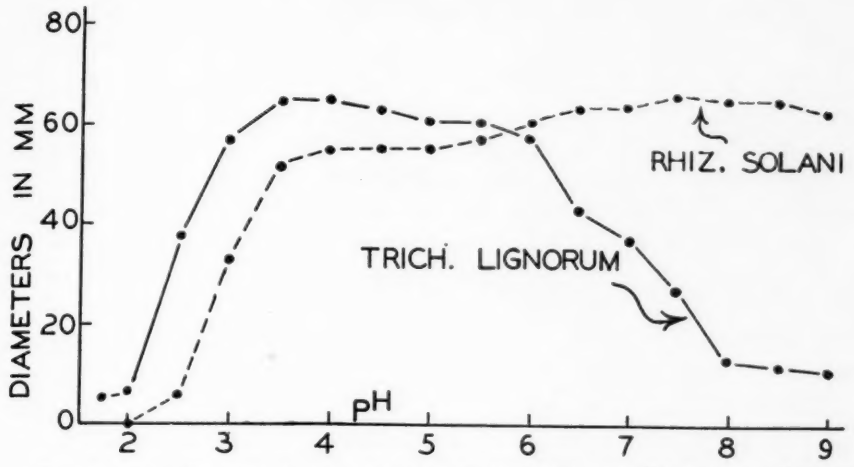


FIG. 6. Diameters of colonies of *Rhizoctonia solani* and *Trichoderma lignorum* grown at 24° C. on a medium containing glucose and peptone for 42 hours at different pH values.

The cultures of the *p* strain showed a rapid decline of parasitic activity at pH values higher than 5.5, although the rate of growth in this range was as rapid as in the range of greatest parasitism. Around pH 5.5 the lethal principle of $P_3$ had also been found to show the greatest increase in deterioration and, subsequently, the greatest loss in effectiveness (Fig. 5). At the marginal pH values the Rhizoctonia hyphae submerged in the medium had a better chance to survive, because the lethal principle may deteriorate before it can diffuse to them. With the host hyphae at the surface of the agar this loss was decreased, the Trichoderma providing for a large production of the lethal principle and its rapid passage onto the Rhizoctonia hyphae by coiling around them or increasing the touching surface in every conceivable way. At the higher pH values coiling occurred occasionally but the Rhizoctonia hyphae were not killed. This indicates that the decomposition of the lethal principle was so rapid that it had no effect.

At pH values lower than 5.5 no living hyphae of Rhizoctonia were found after the attack of Trichoderma cultures $P_2$ and $P_3$. The same result had been obtained in the nonbuffered series (not included in table 9) at an initial pH 3.0 only, the reaction of all the other media having changed sufficiently toward neutrality to allow the survival of at least a part of the Rhizoctonia colony. The culture $P_0$, which appeared to have lost its parasitic ability on ordinary media, was found to produce an amount of lethal principle just sufficient to kill the surface hyphae of Rhizoctonia colonies up to pH 5.0, the submerged hyphae, however, remaining alive (Table 9).

Unfortunately, the lethal index of this culture was not determined because it had been discarded before the standard methods had been developed.

The 2 cultures of the *o* group killed the Rhizoctonia also in media around the neutral point (Table 9). This would explain an observation often made with ordinary culture media of a pH 5.5 and higher, in which the Trichoderma cultures of the *o* group destroy the Rhizoctonia colonies completely, while with the *p* group, sclerotia and living hyphae are found after the Trichoderma attack. Before drawing general conclusions, however, more cultures of both groups have to be examined as to the effect of pH on their action. The lethal principle of the *o* group has not yet been studied in detail. Compared with those of the *p* and *q* groups, it has been found surprisingly weak in preliminary tests made under the standard conditions for $P_3$.

A basidiomycete isolated by D. E. Bliss from the roots of diseased date palms was subjected to the attack of Trichoderma over a range of pH values. The results were similar to those obtained with the Rhizoctonia as a host.

### MISCELLANEOUS EXPERIMENTS

Cultures of *Trichoderma Koningi* Oud. and *T. album* Preuss were secured through the courtesy of the Centralbureau voor Schimmelcultures, Baarn. They were found to attack the Rhizoctonia in the same way as *T. lignorum*.

Attempts towards determining the nature of the lethal principle of the pigmented Trichoderma culture $P_3$ have thus far led to the following results: If the filtrate was passed through a Chamberland B. filter, or concentrated under reduced pressure to a syrup, a part of the lethal effect was retained. A toxic effect on Rhizoctonia was obtained from the residue of ether extracts of the Trichoderma filtrate. Most of the lethal principle seemed to be adsorbed by charcoal when a sterile suspension of it was added to the media together with the Trichoderma spores. Likewise, by adding a suspension of animal charcoal to mixed cultures of Trichoderma and Rhizoctonia, it was possible to suppress the parasitic action of Trichoderma very greatly, without apparently influencing its growth. This was probably not due to an effect on the production of the lethal substance, but rather to its adsorption. This phenomenon may be of importance in connection with attempts to purify the lethal principle and in an understanding of the effect on the parasitic action of soils rich in colloidal material.

Further additions to the host list of Trichoderma are: *Armillaria mellea* (Vahl.) Quel., 2 undetermined basidiomycetes, and *Rhizoctonia bataticola* (Taub.) Butler. No resistance against attack of Trichoderma has been found in a number of strains of *Rhizoctonia solani* from different host

plants obtained through the courtesy of G. R. Townsend, of Cornell University.

An interesting change of pH was noted in the interaction of Trichoderma $O_1$ with Rhizoctonia, when grown on Czapek's agar to which a few drops of brom cresol purple indicator had been added. The dying Rhizoctonia hyphae took up the indicator and, having a more acid reaction than the surrounding purple medium, stained yellow. After some time the acid seemed to diffuse from the hyphae into the medium, rendering it yellow (pH 5.4 and lower), while outside the zone of dead Rhizoctonia hyphae, the medium remained purple (pH 6.4 and higher).

In a similar way the dying Rhizoctonia hyphae seem to accumulate under certain conditions the pigment of Trichoderma and again lose it, as previously described and illustrated (40). No other connection between pigment and parasitic action has thus far been observed. Like the lethal principle, most of the pigment is adsorbed by charcoal, and it may be extracted by ether from the filtrate. The ineffective filtrate from old cultures of Trichoderma of the *p* group, however, contains very much pigment, and the lethal index of culture filtrates of the nonpigmented *q* group is similar in amount to that of the *p* group. This latter group may be identical with the strain of *Hypocrea rufa* Pers. on the yellow pigment of which Medisch (26) has done a considerable amount of work.

Swarm spores of *Phytophthora citrophthora* also have been killed by concentrations of the lethal principle similar to those effective on Rhizoctonia.

## DISCUSSION

The main purpose of the remarks that follow will be to point out some phases of the problems opened up by the work done. Some of the fundamental aspects of the subject having been recognized, further investigation is necessary to gain a more comprehensive view of the problems.

Brown (5) has sufficiently emphasized the importance of uniform methods in obtaining toxic substances produced by fungi at the time of highest parasitic activity. His view is supported by the work reported here. Many so-called facultative parasitic fungi need apparently to acquire a certain vitality through a period of saprophytic growth before they are capable of parasitic activity. With the Trichoderma this transition from the saprophytic to the parasitic habit seems to be dependent on the production of a sufficient amount of lethal principle, which, under the conditions used, took place about $1\frac{1}{2}$ days after germination of the spores. Due to continuous deterioration, the lethal principle of *Trichoderma lignorum* does not accumulate during the growth of the fungus in the culture medium. This makes it possible to secure an approximate curve of the rate of production of the

lethal principle during the life cycle of the fungus (Fig. 2). The true curve of the rate of production would exclude the residual amounts from previous production that have not yet decomposed. The slope of this curve would rise, therefore, somewhat slower and descend more rapidly than is actually shown in figure 2. The possibility that the rapid decline in the curve of production during the third day may be due to a rise in pH, which increases the rate of decomposition of the lethal principle, is excluded by the fact that the pH of the medium did not change to any considerable extent before the fourth day of these experiments.

The nature of the toxic substances produced by parasitic fungi has been found to differ widely. Endoenzymes (5) and exoenzymes (1, 12, 15) have been reported. More or less definite chemical compounds, like nitrites (21, 31), aldehydes (20), or organic acids (17) have been isolated and thought responsible for the toxic effect of the filtrates. Certain organic acids have been found toxic to some fungi at very low concentrations (19). As to the chemical nature of the lethal principle of *Trichoderma lignorum*, no conclusive evidence has been presented. There is, however, one significant property of the lethal principle remaining constant under extremely varying conditions. The ratio between the lethal indices obtained by adjusting the filtrate to certain pH values does not seem to be much affected, whether the lethal principle is secured at different stages of growth of Trichoderma, or obtained as a residue from different degrees of decomposition by boiling and by aerobic or anaerobic storage at different temperatures. This should hardly be expected if it were a mixture of different substances. It seems to indicate that the lethal principle is a single definite chemical substance that is readily oxidized and of nonenzymatic nature. It is possible that this substance is a specific product of metabolism of species of Trichoderma, such as the substances found specific for certain groups of fungi in the excellent work of Raistrick and his collaborators (30). A group of Penicillium species has been found to produce a specific substance, penicillin, which, in very high dilutions, is able to suppress certain bacteria (14). This substance is said to be nonenzymatic and rather unstable.

In the determining effect of pH on the interaction of Trichoderma with Rhizoctonia, the instability of the lethal principle seems to be the deciding factor, although there is no proof that it is the only one. There is, under a definite set of conditions, a certain pH range at which the parasitic action decreases rapidly, no parasitism being exhibited at higher pH values. Apparently, in this range of pH values, the decomposition of the lethal principle proceeds so fast that the amount left becomes smaller and smaller with increasing pH, until it is not sufficient to kill the Rhizoctonia. It is evident that this pH range is not absolutely fixed, but will be influenced to a certain extent by many factors. They may be external, like nutrients,

moisture, temperature, etc.; or they may concern the fungi themselves, such as types, stages of development, and relative amounts of parasitic and host mycelium.

Besides, environmental factors have an indirect influence on the parasitic action, by affecting the growth and development of each of the fungi; *e.g.*, the growth of Rhizoctonia has been seen to be at a greater advantage, as compared with Trichoderma, the more alkaline the medium becomes. A medium of low moisture content is more unfavorable for the growth of Trichoderma than for Rhizoctonia. On the other hand, Falck (11) finds that Trichoderma species are able to get established in water-logged lumber before the dry-rot fungi, and that the later invasion of the wood by the dry-rot fungi is checked more or less by toxic substances excreted by Trichoderma. A similar rôle may be played by Trichoderma species in water-logged soils, in subsoil, and certain forest soils, where it is reported to be abundant (7, 37).

Another important problem is the great difference in aggressiveness of different groups of *Trichoderma lignorum* and other species of Trichoderma. In this connection, the possible rôle of prolonged artificial culture in decreasing the parasitic ability needs to be considered. A wide field is also opened up in the study of resistance against Trichoderma attack by comparing the effect of the lethal principle and of the parasite on the wide range of hosts, which are probably more numerous than has been hitherto reported (3, 27, 40). Such studies also may give a better understanding of some soil-microbiological processes and their relation to the action of plant-pathogenic organisms.

Preliminary experiments on the rôle of the interaction of Trichoderma and Rhizoctonia in the soil have borne out the results of the work in culture. By merely adding a spore suspension of *Trichoderma lignorum* to a sterilized soil at the time of a heavy inoculation with *Rhizoctonia solani,* Citrus seedlings were better protected from damping off at reactions below pH 5.5 than at reactions higher than pH 5.5. Trichoderma culture $O_2$, which was found more effective than $P_3$ in culture, also appeared to be more actively parasitic in soil. These facts explain the variable results of mixed inoculation of soils in former experiments (39, 41). Together with good growth of Trichoderma at acid reactions, the influence of low pH values on the interaction with other organisms may contribute to the prevalence of Trichoderma species in acid soils (36, 37). Bisby and his associates (3) report an experiment in which the Trichoderma has rendered harmless the inoculation of wheat with foot rot fungi in sterilized soil.

Trichoderma species are of universal occurrence as soil organisms. They were shown to be extremely active in the decomposition of soil organic matter (37). The above reports indicate that these fungi, under favorable

conditions, may play an additional beneficial rôle in the soil, reducing plant diseases by attacking soil-borne pathogenic fungi.

Recent work on the antagonism between saprophytic soil organisms and plant pathogens (8, 9, 22, 33) is concerned mostly with surveying the effect of a large number of antagonistic organisms towards a few plant-pathogenic fungi. As a supplement, it seems necessary to single out individual antagonistic organisms, and to study their behavior towards one or more plant pathogens, employing in such an investigation accurately controlled environmental conditions.

## SUMMARY

A lethal principle has been found to be instrumental in the parasitic action of *Trichoderma lignorum* on *Rhizoctonia solani* and other soil fungi.

Uniform procedures have been developed for obtaining and testing the lethal principle of the pigmented culture of *Trichoderma lignorum*. In order to compare the lethal effects of various toxic solutions, the numerical expression "lethal index" is employed. The lethal index is represented by the reciprocal of the minimum concentration at which the Rhizoctonia hyphae are killed.

The approximate curve of production of the lethal principle during the life cycle of Trichoderma indicates that the lethal principle is excreted into the surrounding medium by young hyphae only. There is evidence suggesting that the lethal principle is a single chemical substance. It seems to be neither of enzymatic nature nor a result of autolytic processes.

A characteristic property of the lethal principle is its rapid decomposition. Under aerobic conditions, the rate of deterioration increases with increasing pH. This process is much slower at 2° C. than at room temperature. Anaerobic decomposition of the lethal principle, however, is rather independent of the pH of the medium, and does not seem to go to completion. Boiling of the filtrate greatly weakens the lethal principle, but even prolonged autoclaving does not destroy it completely.

If portions of the filtrate containing the lethal principle are adjusted to a series of pH values, the lethal effect is found to decrease with increasing pH. This is believed to be due to the more rapid decomposition at the higher pH values.

The effect of the lethal principle of *Trichoderma lignorum* and the action of the fungus itself on *Rhizoctonia solani* parallel each other in a number of ways. (1) The maximum amount of lethal principle is produced 2 days after the germination of the Trichoderma spores. Likewise, the strongest parasitic action occurs at that time. (2) The effectiveness of the lethal principle has been found to decrease most rapidly in the pH range between pH 4.8 and 6.3. In approximately the same range the pigmented

cultures of *Trichoderma lignorum* gradually lose their ability to parasitize the Rhizoctonia, no attack taking place at alkaline reactions. (3) If an adsorbing agent, such as animal charcoal, is added on sowing the Trichoderma spores, filtrates very poor in lethal principle are obtained, and, in the presence of the Rhizoctonia, the parasitic action of Trichoderma is greatly inhibited.

The group characteristics and the effect of prolonged artificial culture of *Trichoderma lignorum* seem to play a great rôle in the amount of lethal principle produced and in the pH range of parasitic action. Cultures of the fungus that are characterized by a cocoanut-like odor have been found to attack the Rhizoctonia at neutral and alkaline reactions at which no parasitism is exhibited by pigmented cultures.

A comparison of the pH growth curves of *Trichoderma lignorum* and *Rhizoctonia solani* shows that alkaline reactions are favorable to Rhizoctonia but unfavorable to Trichoderma.

Good control of damping off of Citrus seedlings due to *Rhizoctonia solani* has been secured by adding Trichoderma spores to a sufficiently acid, sterilized soil.

*Trichoderma Koningi* and *T. album*, as well as all isolations of *T. lignorum* tested, have been found to attack the Rhizoctonia.

*Trichoderma lignorum* also is capable of parasitizing *Rhizoctonia batatícola* and *Armillaria mellea*.

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# SPECIFIC QUANTITATIVE NEUTRALIZATION OF THE VIRUSES OF TOBACCO MOSAIC, TOBACCO RING SPOT, AND CUCUMBER MOSAIC BY IMMUNE SERA

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Chiefly through the studies of Beale (9, 10) the attention of a number of investigators has recently been directed to an analysis of the possible antigenic capacity of the plant viruses. It has been known for a number of years that the animal viruses are capable, in general, of engendering in inoculated susceptible and nonsusceptible animals the production of specific neutralizing antibodies, and possibly of specific precipitins as well. From the works of Beale and their confirmation and extension by Matsumoto (5), Silberschmidt (11), Gratia (2), and Birkeland (1) we know that the inoculation of rabbits with preparations containing tobacco-mosaic virus results in sera that precipitate such virus preparations at dilutions of 1:100 or higher, that the reaction in question occurs regardless of the host species harboring the virus, so far as has been tested, that the reaction occurs without loss of precipitin titer even though the virus has been subjected to purification by various methods, and that the reaction appears to be specific for the virus used as inoculum. It thus would appear that the antigenic substance involved is either the virus itself, or else some close combination of virus with other substances present in the plant juices.

Up to the present no satisfactory evidence has been advanced that serum immune from a plant virus is capable of neutralizing or inactivating that virus. Beale (9) and Silberschmidt (11) have used the term "neutralization," referring to their studies on the inhibition of tobacco-mosaic virus by serum; but in these works it was not made clear whether this inhibition operates through an effect on the virus (neutralization) or through a lowering of the susceptibility of the host plant. To be sure, the term neutralization is used by animal serologists, although a possible effect on the host might be conceived. Yet the methods of inoculation differ so markedly in the two fields that this practical source of error with the plant viruses may have but slight theoretical importance as regards the animal viruses. Holmes (3) has shown that toxic materials in tobacco sap will decrease tobacco-mosaic infectivity through a deleterious effect on the host. Recently, Stanley (12) pointed out that trypsin likewise reduces the infectivity of tobacco-mosaic virus not by affecting the virus but by an effect on the host plant. Thus it is evident that before the term neutralization may

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be applied to the action of serum when mixed with virus preparations, it is first necessary to determine whether or not the effect observed is actually an effect on the virus involved.

Since neutralization is necessarily an effect, direct or indirect, on the virus itself, not primarily involving the non-virus constituents of virus preparations, it has been felt that an investigation of the ability of plant viruses to induce in animals the production of neutralizing antibodies might add instructive evidence to that obtained from a study of the precipitin reaction. One of the ultimate ends of serological studies with the plant viruses is a conclusion as to the fact and extent of the antigenicity of these viruses; but, since the studies here reported are being continued, it is felt advisable to postpone an analysis of the whole question of plant-virus antigenicity until more evidence is in hand. The present paper is accordingly confined to a report of investigations of the neutralization of certain plant viruses by immune serum.

## LITERATURE

In 1926 Mulvania (6) first published an account of researches involving the inhibitory effect of rabbit serum on the virus of tobacco mosaic. Mulvania found that the addition to virus preparations of fresh, normal rabbit serum caused a decrease in infectivity of the virus. The infection experiments were performed by inoculating ten tobacco plants with each of the serum-virus mixtures. Two years later Purdy (9), using a similar technique in a more extensive series of experiments, confirmed Mulvania's finding and reported further that serum of rabbits immunized with tobacco-mosaic extract is more effective than normal serum in reducing the percentage of infectivity of serum-virus mixtures. Matsumoto in 1930 (5) again noted the inhibitory effect on tobacco-mosaic virus of antiserum, and in 1933 Silberschmidt (11) confirmed the accounts of these previous investigations in showing that mixtures of virus and antiserum, if plated out on leaves of *Nicotiana glutinosa*, produce fewer local lesions of the tobacco-mosaic disease than do corresponding mixtures of normal serum and virus, although the latter mixtures showed some decrease in infectivity as compared with serum-free controls.

From the works cited one is justified in concluding that the addition of normal rabbit serum to tobacco-mosaic virus causes a decrease in the infectivity of the virus preparation, and that the addition of mosaic-immune serum causes a still further decrease in infectivity. Beyond this one must proceed with some reservation in analyzing these earlier works, since any error due to host effect was not considered, and since, in a large part of the work reported, the method used for determination of virus infectivity (pin-prick inoculations of a few tobacco plants) was relatively insensitive, as

compared with the more efficient method of plating virus on local-lesion hosts.

Preliminary to the present study, the findings of the four investigators mentioned above were confirmed, and it was found that by the use of comparable techniques the inhibiting effect of normal serum and the greater inhibitory effect of antiserum were readily demonstrated.

The question now arises as to whether the relatively weak inhibitory effect of normal serum and the stronger inhibitory effect of immune serum are qualitatively different. The problem resolves itself into a determination of whether the inhibitory effect of serum is due to a decrease in the susceptibility of the host or to an inactivation of the virus. An effect on the host would serve to explain the results thus far recorded, but only a specific, quantitative effect on the virus itself would justify the use of the serological term neutralization.

## MATERIALS AND METHODS

*Virus stocks.* Viruses of the following types and sources, all propagated in plants of *Nicotiana tabacum* var. Turkish, served as infective material for the experiments reported:

a. *Tobacco mosaic:* A strain that had been repeatedly purified by single-lesion isolations was obtained from J. H. Jensen.
b. Several brilliant strains of tobacco mosaic isolated by J. H. Jensen (4) and obtained from him. Of these brilliant strains those particularly employed in the present study were "Jensen 108," a white mottling strain, which causes very severe distortion and stunting, and "Jensen 306," a moderately yellow variant.
c. A symptomless strain of tobacco mosaic obtained from F. O. Holmes.
d. *Tobacco ring spot:* An unpurified strain of common green type.
e. *Cucumber mosaic:* A green strain (Porter's No. 1), and a brilliant yellow strain obtained from W. C. Price and designated by him as No. 6 (8) both of which he had repeatedly purified.

*Virus Preparations.* The following types of virus preparations were successfully used in the experiments to be reported:

a. Expressed sap of diseased plants either used at once after expression, as was always done with the viruses of cucumber mosaic and tobacco ring spot, or kept frozen until needed (virus of tobacco mosaic and its strains).
b. Extracts of fresh diseased tissues macerated in two parts of physiological saline solution, cleared, and kept frozen until needed (tobacco mosaic and its strains).
c. Tobacco-mosaic virus purified by basic and neutral lead acetate precipitations (13) and kept frozen until needed. This type of preparation was used most extensively with tobacco mosaic because of its high infectivity and relative freedom from non-virus materials.

*Host Plants.* Infectivity of virus samples and virus-serum mixtures was determined by counts of the local lesions produced on the primary leaves of the bean, *Phaseolus vulgaris* L. var. Early Golden Cluster, and occasionally, on young leaves of *Nicotiana glutinosa* L., in the case of tobacco mosaic. Cucumber mosaic and tobacco ring spot were similarly titrated on the primary leaves of the cowpea, *Vigna sinensis* (L.) Endl. var. Black Eye, according to techniques described by Price (7, 8).

*Technique of Inoculation of Host Plants.* Inoculation for the propagation of stock virus material was by the pot-label method (4).

Inoculation of beans and cowpeas for infectivity counts was by uniformly rubbing leaves with a piece of folded gauze moistened with the solution to be tested. Rigorous care was taken to prevent accidental infections, high dilutions always being plated out before low dilutions, hands being thoroughly washed between inoculations of any two different solutions, and the inoculated leaves being rinsed a few minutes after inoculation.

*Preparation and Care of Sera.* Rabbits, weighing approximately 1500 gm., were used exclusively for the production of sera. Eight injections at 3- or 4-day intervals were employed, the customary practice being to inject 2 cc. intraperitoneally for the first injection, followed by 7 intraperitoneal injections of 5 cc. each. Intravenous injections of similar amounts of inoculum at similar intervals were employed in preliminary work, but the added labor and danger was not compensated for by any superiority in titer of the intravenous sera. Of 35 animals immunized only 3 were lost, 1 from coccidiosis, 1 from shock following an intravenous injection, and the third apparently from septicaemia.

In preliminary experiments virus solutions for injection were prepared by centrifuging 1 : 2 saline extracts of fresh virus tissues,—later, and in the greater part of the work here reported, centrifuged expressed saps were employed, the expressed saps giving somewhat more potent immunization than the extracts.

Five to 10 cc. of normal serum was taken from each animal prior to immunization. This serum was frozen and used as control normal serum for the immune serum subsequently obtained from the same animal. Nine or 10 days following the 8th inoculation, after 24 hours without food, the animals were anaesthetized with ether and bled from the carotid artery. The blood was allowed to clot 2 hours at room temperature and over night at a temperature a little above freezing, then the serum was decanted, centrifuged twice, and kept frozen solid until needed. Ordinarily there was little or no sign of deterioration as regards either neutralizing or precipitating power, even in sera that had been several times momentarily melted and then refrozen, although in two or three exceptional cases sudden loss of titer was observed. Sera used 6 months after the original freezing, a

period interrupted several times by partial melting, were usually fully as potent as originally.

*Serum-virus Mixtures.* In preparation of a neutralization experiment a series of dilutions of virus were prepared, the dilutions being in physiological saline, Ringer solution, or M/10 $K_2HPO_4$. To each sample of each dilution of virus was then added a constant amount of normal or immune serum-dilution, the sera being diluted in the same solvent as the virus. A series of virus dilutions mixed with the serum diluent was used in every case as a control. Wherever possible the immune and normal sera used in any given experiment originated in the same animal. With cucumber-mosaic and tobacco-ring-spot viruses the highest virus dilution practical was 1:100; with tobacco mosaic, infections have been obtained in virus stocks diluted 1:10,000,000, although 1:20,000 and 1:200,000 were the highest dilutions employed in routine work. The sera tested against cucumber mosaic and tobacco ring spot were invariably used at a net dilution of 1:50 (2 normal drops of a $\frac{1}{5}$ solution being added per 1 cc. of virus dilution). With tobacco mosaic the sera were usually diluted 1:200 (1 cc. of 1:100 serum dilution per 1 cc. virus dilution), although recent work has shown that serum dilutions of 1:1000 or higher are even more satisfactory when it is desired to minimize the inhibitory effect on virus of normal serum, and when relatively potent virus preparations are used.

It has been found advisable to absorb all sera, normal and immune, with 2 parts of healthy tobacco sap, for 2 hours at 37° C., followed by centrifuging and the making of proper allowance for dilution by the healthy tobacco sap. This has proved advisable, since even normal serum produces a slight precipitate with tobacco-mosaic virus preparations, particularly when the serum is in excess, and this precipitate mechanically removes from suspension a certain amount of virus, which tends slightly to distort the results.

Mixtures of tobacco-mosaic virus and absorbed serum were repeatedly shaken during an incubation time of 1–2 hours at room temperature, then plated out directly. With the viruses of cucumber mosaic and tobacco ring spot this time was usually reduced to $\frac{1}{2}$–1 hour. Incubation at 37° C. is unnecessary and fails to intensify appreciably the inhibitory effect of anti-serum.

*Care of Apparatus.* All glassware was cleaned with a dichromate-$H_2SO_4$ solution and thoroughly rinsed before using. Further sterilization was found to be unnecessary. The same treatment was applied to the porcelain mortars used in maceration. Hypodermic needles were cleaned in strong boiling sodium bicarbonate solution, then washed and sterilized by boiling. Gauze inoculation pads were prepared from new gauze and sterilized in steam before using.

*Infectivity Counts.* With the cowpea, the number of local lesions pro-

duced per leaf is never too great to be counted with ease. With the bean, the number of lesions per leaf frequently reaches 1600 or more. In such cases it was customary to count accurately all leaves with less than 50 lesions, while leaves bearing more were estimated as to number by comparing with a plaque of dried bean leaves bearing a series of counted numbers of lesions. With tobacco mosaic, it was customary to use 16–18 leaves for each mixture containing virus at less than 1:2000 dilution, and 32–72 leaves for more dilute samples. With cucumber-mosaic and tobacco-ring-spot viruses, 20 leaves were used for dilutions below 1:10 and 30–80 leaves for more dilute samples. Whenever a strong inhibitory effect was anticipated, such as to decrease the count markedly, as with tobacco-mosaic virus and tobacco-mosaic antiserum at virus dilutions below 1:2000, correspondingly greater numbers of leaves were employed. Twelve dozen pots of plants (750 culled leaves of bean, 1000 culled leaves of cowpea) were customarily used for each experiment involving the testing of 3–4 virus-serum mixtures with controls at various dilutions. Each experiment reported has been repeated at least once with confirmatory results. The experiments on occurrence of the neutralization reaction and on its specificity have been repeated many times with no essential departure from the results indicated below.

*Plan of Experimentation.* Since, as has been pointed out above, it was first necessary to determine, if possible, whether the inhibitory effect of normal or immune serum is an effect on host susceptibility or on virus, the following type of experiment was devised for differentiating these two effects. If the serum effect is on the susceptibility of the host, then a given amount of serum would be expected to produce the same percentage of decrease in susceptibility regardless of the amount of virus simultaneously applied; if, on the other hand, the effect of the serum is on the virus itself, then a relationship would be expected to exist between serum and virus such that a given amount of serum would hardly have a detectable inhibitory effect if an excess of virus is present, while as the amount of virus is decreased, the amount of serum being held constant, the relative inhibitory effect would become proportionally greater. From the data obtained from such an experiment, if the percentage of infectivity as compared with the serum-free virus control at each dilution be plotted (ordinate) against degree of virus dilution (abscissa), then one might have a means of differentiating the host-effect from the virus-effect, the former yielding a horizontal line, depressed below the control (100 per cent infectivity) to an extent determined by the constant absolute amount of serum added, the latter giving a curve essentially coincident with the control horizontal where the amount of virus is in excess of the amount of serum, but rapidly curving downward as the amount of virus approaches the amount that can be completely neutralized by the constant amount of serum employed.

Such an experimental arrangement was accordingly attempted, and, preliminary tests having shown its suitability, the same arrangement with such modifications as were necessary in the elucidation of various points, has been made the basic type of experiment in the present investigation.

## EXPERIMENTATION

### The Characteristic Effects of Normal Serum and Immune Serum on Virus Infectivity

Normal serum and tobacco-mosaic immune serum, each at net dilutions of 1:200, were respectively mixed in constant amounts with a dilution series of lead acetate purified tobacco-mosaic virus, and the results of infectivity counts were plotted in terms of the infectivity of the serum-free control, as was projected above. The results are illustrated in figure 1, which represents one of many such experiments.

It is seen from the figure that normal serum has an effect of lowering the percentage of infectivity by essentially the same degree throughout the whole of the experimental range of virus dilutions, from 1/2 to 1/20,000, and with a variability relatively low for a biological test. The only point that fails to conform well to a parallel position for the two curves is the first, at virus dilution 1/2. The explanation of this single abnormally high point, which is characteristic for the curve wherever tested, is apparent when one considers the effect of dilution of the virus alone. From dilutions 1/2000 to 1/20,000 the drop in infectivity for the normal curve as given in absolute terms above the curve is approximately proportional to the factor of 10 used in this step of dilution. As we proceed to the left, however, there is less and less proportionality until from 1/2 to 1/20 the number of lesions is decreased by only a little more than half. This flattening of the dilution curve of tobacco-mosaic virus in the higher concentrations of virus is due to two elements, one of which, as will be seen below, is an effect of the normal constituents of tobacco juice on the host plant, an effect that in this case was at a minimum because a purified virus preparation had been used; while the other element may be termed the saturation of the bean tissues for virus at high concentrations. The mean number of lesions per leaf of the control virus at 1/2 was 1220. It would be easy to double the concentration of this virus, yet more than 1600–2000 lesions have never been seen on a bean leaf. Thus at very high concentrations of virus the limit is approached at which the amount of virus can be no longer proportionally registered by the number of lesions appearing. Some lesions at these higher concentrations are doubtless caused by more than one infective unit of virus. Hence the absolute number of lesions approaches a maximum beyond which it cannot pass regardless of the concentration of virus, a limit conditioned by the ability of the bean leaf to register lesions. The

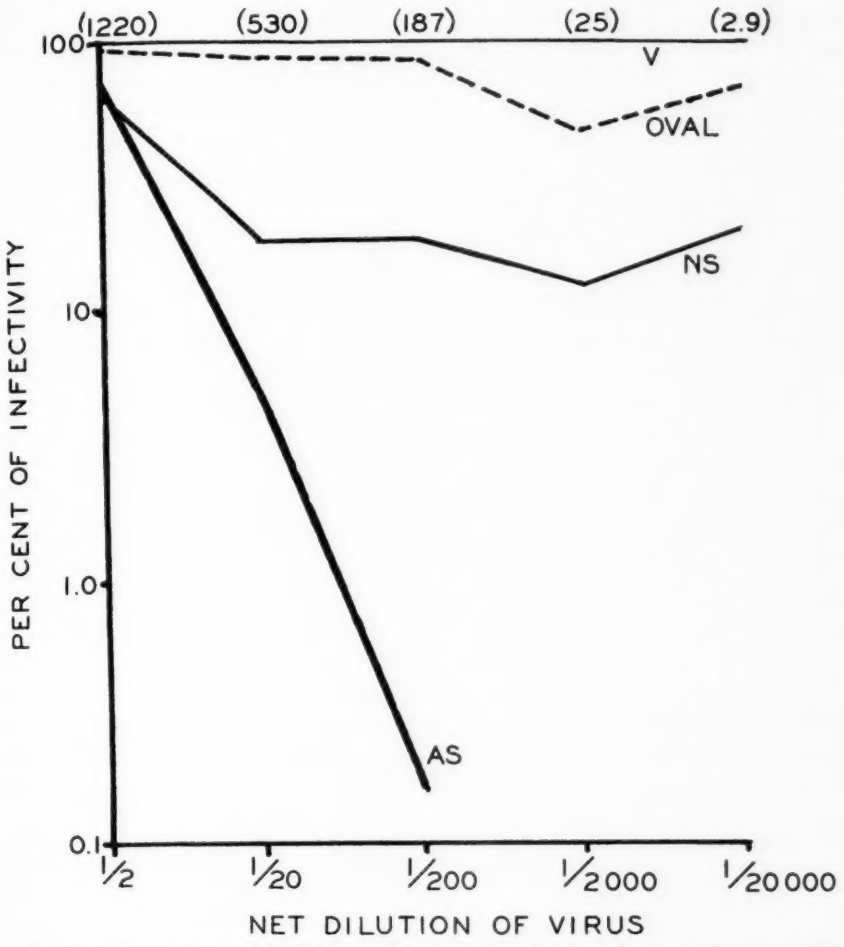


FIG. 1. Percentage of inhibition of tobacco-mosaic virus by normal serum (NS) at 1: 200, by anti-tobacco mosaic serum (AS) at 1: 200, and by ovalbumin (Oval) at 1: 200, all in terms of infectivity of untreated virus control (V). Percentage of inhibition determined by infectivity counts on bean. In this and the graphs to follow, the absolute values for the control curve, V, are given in parenthesis above each point of the dilution scale, and from these absolute values the value of any point on any curve can readily be computed. Further discussion in text.

number 1220 of our experimental virus at 1/2 is not far from that maximum. Meanwhile, the serum-treated samples at the same dilution gave only about 2/3 that number of lesions, *i.e.*, with them the depressing effect due to saturation of the bean tissues for virus was but 2/3 or less that of the control, other factors being equal. Thus the first points for the normal serum and antiserum curves are proportionally higher than they should be

on the basis of pure serum effect alone. No correction has been attempted for this error, which is not usually observed beyond the first or second points of the dilution scale, but an appreciation of its nature and significance furthers an interpretation of the essential parallelism of the normal serum and control curves, a parallelism that is interpreted as indicating that normal serum decreases the infectivity of the virus involved through a lowering of the susceptibility of the host to the virus, not through any detectable effect on the virus itself.

Passing now to the immune serum curve, we observe that it starts from a point essentially coincident with that of the normal serum curve at 1/2 virus dilution; and then, as the absolute amount of virus decreases, the immune serum curve rapidly falls off, fast approaching zero at a virus dilution of 1:200, a result attributed to a direct or indirect effect of immune serum on the virus itself. If the host-tissue saturation error as applied to the normal serum curve also be applied in the same way to the immune serum curve, the first portion of the latter will be much more nearly horizontal, and the curve will tend to approximate even more closely the theoretical curve for a substance affecting virus. As one might expect, antiserum has in addition the same host effect as normal serum, hence it approaches the horizontal position of the normal serum curve as a limit, not that of the control virus curve.

It will be noted that the aspecific host effect of normal serum is essentially of the same type as that of ovalbumin (figure 1). This same phenomenon has been tested for a number of inhibitory substances unrelated to the virus, *e.g.*, milk, normal tobacco extract, and found in each case to be the same type as the effect due to normal serum. The egg-albumin curve resulted from the use of this protein at a dilution of 1:200. Tobacco extract, on the other hand, must be used at relatively high concentration (1:5 or less) to display a corresponding effect. The fact that tobacco extract has thus been shown to have an effect in lowering the susceptibility of the bean to tobacco-mosaic virus demonstrates the validity of regarding the presence of tobacco products in virus saps and extracts as one of the factors (see above) involved in the flattening of the normal curve for tobacco-mosaic dilutions tested for infectivity on bean leaves.

Turning next to the question of the inherent variability of the reactions described, reference to figure 2 shows at a glance a repetition of the same type of experimentation applied to a number of types, strains, and separate preparations of virus, using different lots and species of test plants, different normal and immune sera at different dilutions, different diluents, and performing the reactions at different times. Consistently throughout one sees that, although there is variation in slope and point of origin of the descending curve for immune serum and in the smoothness of the parallel-

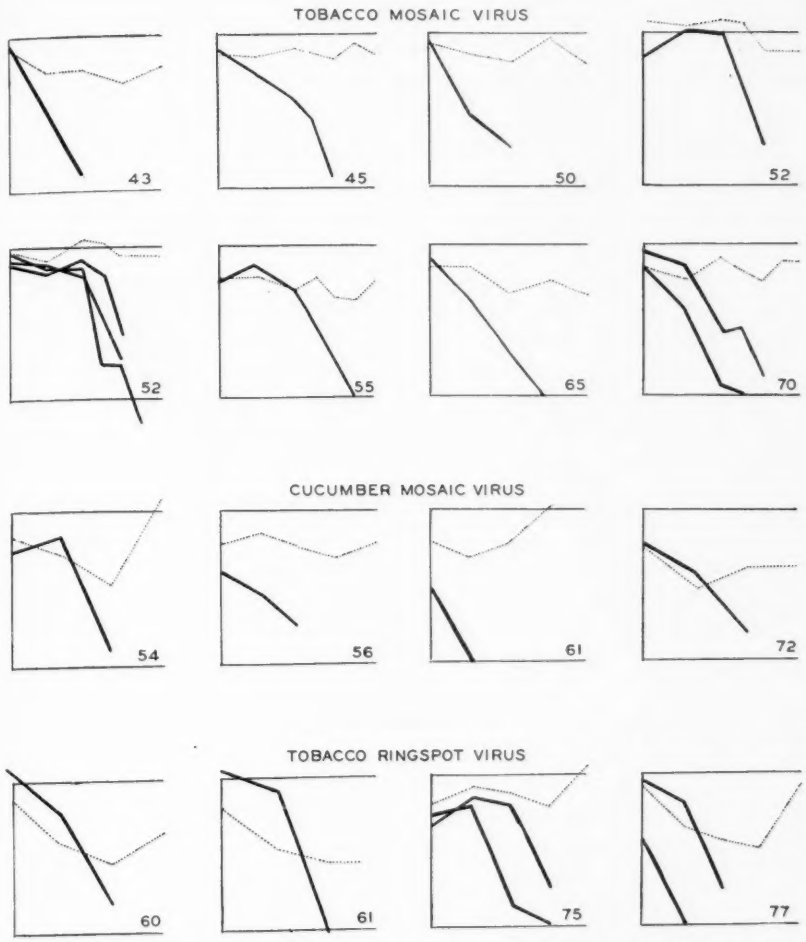


FIG. 2. Examples of normal serum and immune serum inhibitions of tobacco-mosaic, cucumber-mosaic, and tobacco-ring-spot viruses. In each case the upper solid line represents the virus control, the dotted line the normal serum reaction, the heavy line the immune serum reaction Abscissa, virus dilution; ordinate, percentage of infectivity in terms of virus control. The numbers refer to the protocols involved. Further discussion in the text.

ism of the normal serum curve, there is a constant tendency for normal serum to lower the control curve to a greater or less extent, while roughly preserving its horizontal character, and for the immune serum curve to maintain the parallelism with the control for only a short distance before commencing an abrupt fall.

Since it has been noted that the immune serum curve has a tendency to parallel the control curve for some distance before its rapid fall (see

Experiments 52, 55, and 75 in Fig. 2), the question might arise as to whether the normal serum curve is essentially different from the antiserum curve, whether the normal serum curve is merely parallel to the control curve

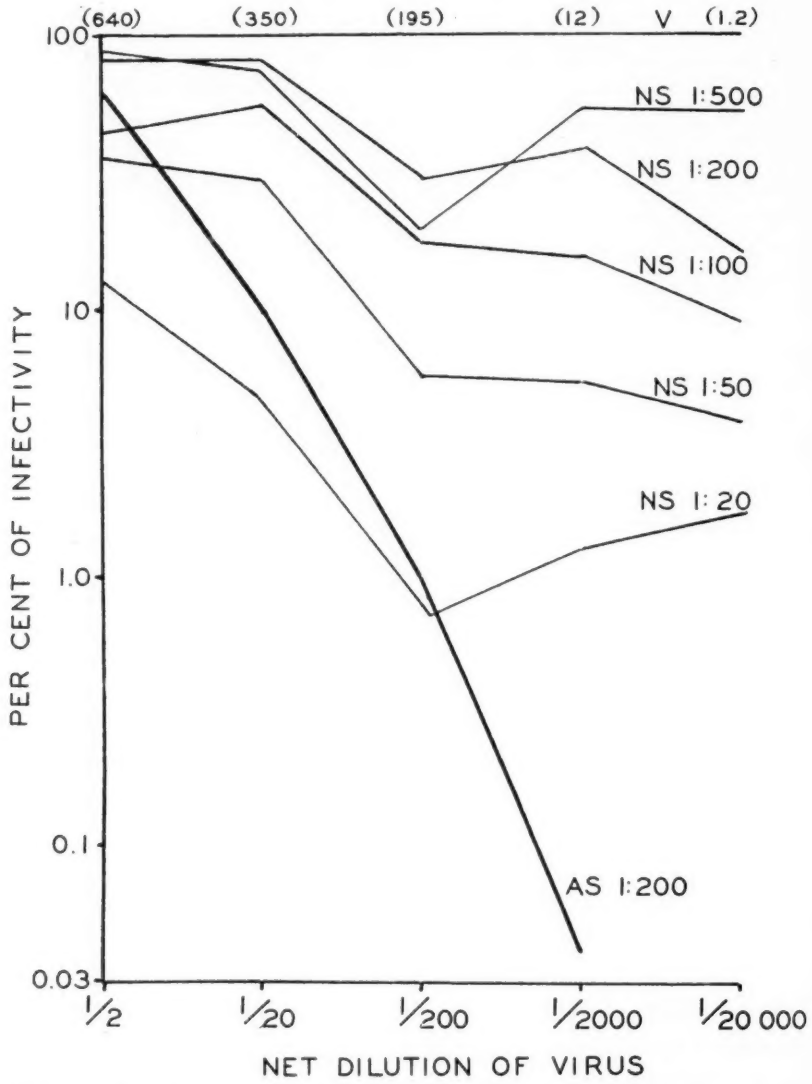


FIG. 3. The effect on virus infectivity of varying the concentration of normal serum. Normal serum (NS) at dilutions as indicated, immune serum (AS) for comparison, all in terms of control virus (V), the absolute values of which are given above. Percentage of inhibition as determined by infectivity counts on bean. Discussion in the text.

because the point of its rapid fall has not been reached in the dilutions of virus thus far used; in a word, whether the reactions of immune serum and normal serum are not inherently the same, both being effects on the *virus*, but differing in degree. The possibility appears reasonable, because, as will be shown below, a decrease in the amount of antiserum used lengthens the horizontal portion of the antiserum curve before its abrupt descent, although the ultimate descent appears inevitable. An experiment was performed in order to demonstrate whether or not the normal serum curve retains its parallelism to the limits of experimental possibility.

In the experiment in question the effect of normal serum was tested in concentrations far exceeding those used in routine practice. The results are shown in figure 3. Here one sees that even if normal serum is used in concentration ten times as strong as in previous experiments, the curve resulting is still essentially parallel to the control curve. It may be noted that as the concentration of normal serum increases and the absolute number of lesions produced consequently decreases, the steepness of the slope

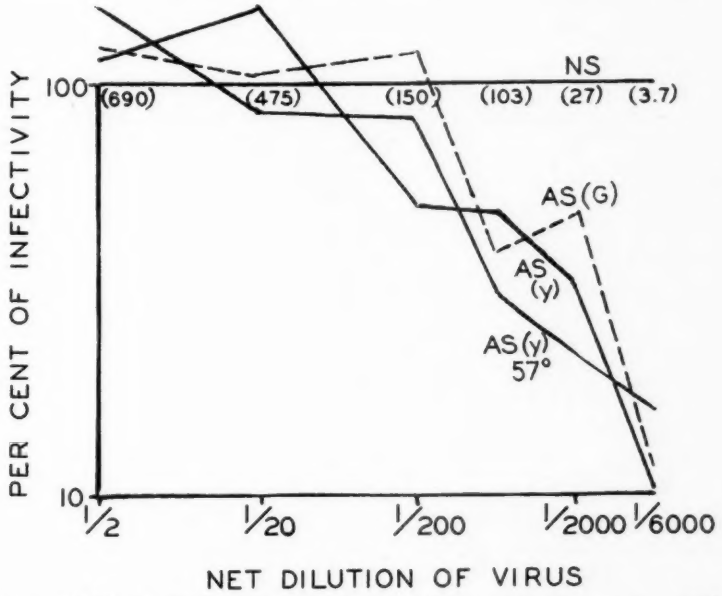


FIG. 4. Effect of complement and of variation in strains of virus employed on neutralization of tobacco-mosaic virus by immune serum. Infectivity of immune serum (AS) expressed in terms of virus plus normal serum (NS) at same dilution, as determined from infectivity counts on bean. Discussion in the text. The virus employed was a white strain of tobacco mosaic, the two antisera were respectively: AS · Y, an antiserum to the same white strain as was used in the experiment; AS · G, a heterologous antiserum immune from the ordinary green strain of tobacco mosaic. Illustrative of the lack of participation of complement in neutralization, and of nonspecificity as between different strains of the same virus type.

due to saturation of leaf tissues with virus increases, which is in conformity with the above interpretation of this portion of the curve.

A further point to consider regards the rôle of complement in the reaction that we may now consider the neutralization of virus by immune serum. Miss Purdy (9), using a technique less sensitive than the one used in the present work, reported that immune serum inactivated for ½ hour at 57° C. was less active in inhibiting the infectivity of tobacco-mosaic virus than unheated serum, and that this loss in inhibitory power was restored on the addition of serum-complement. In order to test this point an experiment was performed in which a portion of antiserum was heated to 57° C. for ½ hour before testing for inhibitory effect. As will be seen from the accompanying graph, figure 4, one observes no essential difference in inhibitory power of the heated as compared with the unheated serum.

This lack of participation of complement in the neutralization reaction of immune serum is consistent with the findings as regards the animal

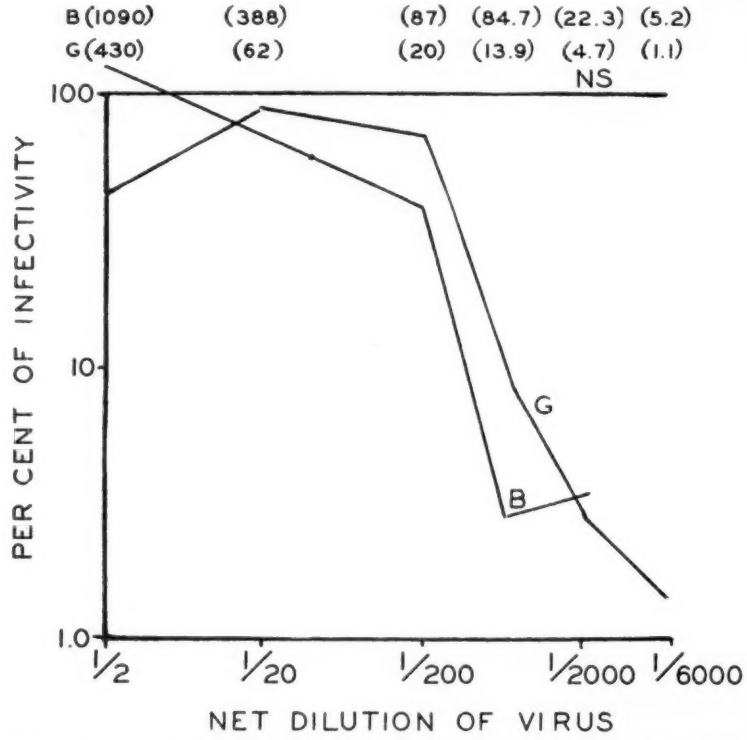


FIG. 5. Percentage of inhibition of tobacco-mosaic virus by anti-tobacco mosaic serum at 1: 200 in terms of normal serum control (NS) as measured by infectivity counts of the same preparations on beans (B) and on *Nicotiana glutinosa* (G). Discussion in the text.

viruses. There is strong evidence in the literature that complement is in general unnecessary for the demonstration of neutralization, as for example in connection with the viruses of rabies and vaccinia.

If, as has been indicated, the inhibitory effect of normal serum is on the host plant, while immune serum in addition has an effect on the virus, further information might be obtained by studying the behavior of the same serum-virus mixtures when tested on different types of host plants. Figure 5 gives the results of such a test, the host plants used being the bean and *Nicotiana glutinosa*, two plants widely separated in systematic position. It will be seen from the curves obtained that the action of immune serum in inactivating virus is essentially the same on both of these plants, which is indicative that we are dealing with a reaction that occurs *in vitro*.

Briefly stated, the present section indicates that the inhibitory effects of normal serum and immune serum are qualitatively different, that normal serum shares with egg albumin, milk, and tobacco extract the quality of affecting the host plant in such a way as to lower its susceptibility to virus to an equal fractional extent regardless of the amount of virus applied. Immune serum, on the other hand, beside sharing this host-affecting property of normal serum, also has an effect on the virus, inactivating it partially or completely, according to the proportions of virus and serum present.

### Quantitative Relationship of Serum to Virus

If antiserum reacts directly on virus, inactivating the latter, then it should be possible to demonstrate a quantitative relationship governing the reaction. Such has been done with respect to tobacco-mosaic virus, and a typical experiment is shown in figure 6. In this case a lead acetate purified supply of ordinary tobacco-mosaic virus was employed in dilution series and to each dilution was added a constant amount of antiserum dilution, the dilutions employed being 1/20, 1/200, and 1/2000. Normal serum at 1/200 was used for control of the customary host effect, and the percentages of infectivity of the mixtures as compared with the virus control plotted as in the figure. The horizontal portion of each curve represents the portion in which there is such an excess of virus that neutralization has not yet become evident, and hence the difference between this horizontal portion and the virus control gives a measure of the pure host effect of all sera. This effect is greater as the concentration of the serum increases, as previously noted in figure 3. The parallelism of the three curves is strong evidence for the occurrence of the same degree of reaction when neutralization finally appears, and the point of this appearance gives an index of the point at which the virus quantity has been sufficiently reduced to show neutralization in the presence of the amount of serum added (supplementary experimentation has shown that the curve for antiserum at 1:20 be-

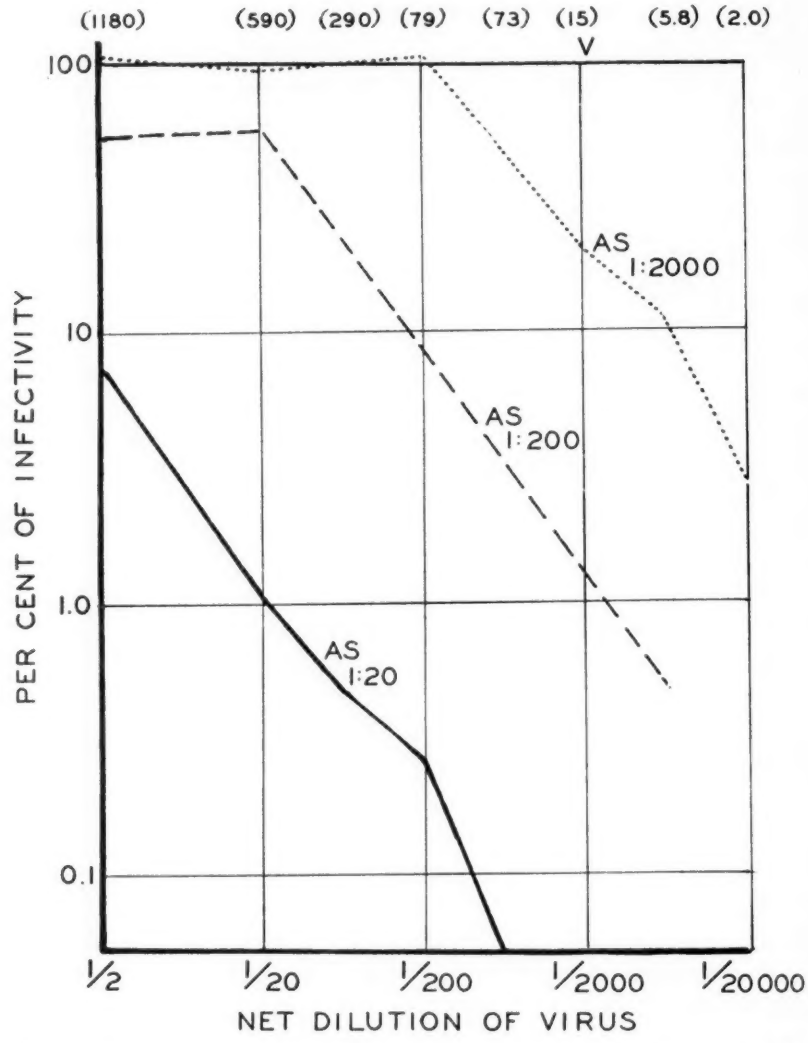


FIG. 6. Quantitative relationship of anti-tobacco mosaic virus serum to tobacco-mosaic virus. Antiserum (AS) at dilutions as indicated compared in infectivity with serum-free control (V). Arrangement as in preceding graphs. Discussion in the text.

comes horizontal at or near the virus dilution 1:2). A comparison of the points of departure of the curves from the horizontal shows that if the concentration and hence the absolute amount of the serum be increased tenfold, then the reaction first appears when the amount of virus present is tenfold, and if the amount of serum be reduced by a factor of 10, then it is able to neutralize only 1/10 as much virus, *i.e., the amount of virus neutralized varies directly as the amount of immune serum added.* If the amount

of virus be doubled, twice as much serum must be added in order to produce the same neutralizing effect. This is a phenomenon entirely independent of any effect on the host plant, since in each curve the relationships of serum to host plant have been maintained constant, only the quantity of virus being varied. This experiment has been repeated using different serum dilutions, and with essentially the same results. We thus see that the interaction of immune serum with tobacco-mosaic virus is governed by a law of simple proportionality, which fact may be best interpreted as indicative that we are dealing with a true reaction on the virus.

### The Time Factor in the Inhibition of Tobacco-Mosaic Virus by Normal and Immune Sera

With neutralization of the animal viruses by specific sera, a certain time of incubation of the serum-virus mixture is customarily considered necessary for adequate demonstration of the neutralizing action. With the plant material at hand it was accordingly of interest to determine the part played by incubation time, if any, on the two reactions before us. Preliminary experiments having indicated that in even so short a time as 5 minutes the neutralizing effect of immune serum is readily demonstrated, it seemed desirable to center attention on even shorter intervals. Accordingly the following experiment was performed.

A highly potent purified preparation of tobacco-mosaic virus was subjected to the action of the most potent immune serum available, and at the same time to that of the normal serum originating in the same animal. One set of virus dilutions was mixed with equal amounts of normal and immune serum, respectively, incubated at room temperature for 1 hour, then plated out on bean leaves, which latter were thoroughly washed exactly 1 minute after inoculation. A second set of the same virus dilutions was mixed with equal amounts of the same sera, incubated at room temperature for 4 minutes, then plated out on beans, the leaves of which were also washed after 1 minute. A third set of the same serum-virus mixtures were shaken thoroughly after mixing and inoculated at once, the leaves being washed after 1 minute as before. In the case of these last, momentary mixtures, in order to avoid too much delay between mixing and inoculation, several separate identical mixtures were successively used whenever the number of pots of beans to be inoculated exceeded 3. The time between inoculation and washing was controlled by a stop-watch. The inoculation of 3 pots of beans required about 2 minutes, hence this represents the maximum time consumed in the case of the "nonincubated" mixtures. The three lots thus were incubated respectively: 1 hour $\pm$ 1 minute; 5 minutes $\pm$ 1 minute; and 1 minute $\pm$ 1 minute; and were all washed exactly 1 minute after the inoculation.

The results of this experiment with regard to the immune sera are given in figure 7. It is seen that if the incubation time is shortened from 1 hour

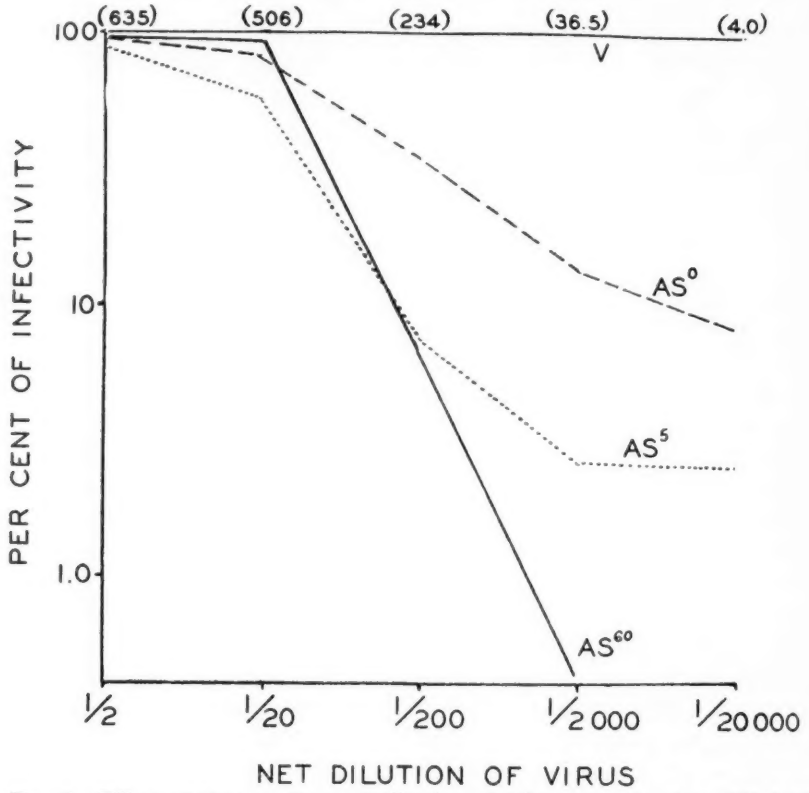


FIG. 7. Effect of time on the neutralization of tobacco-mosaic virus (V) by immune serum (AS). $AS^0$: Serum-virus mixture plated without incubation; $AS^5$: Serum-virus mixture incubated 5 minutes at room temperature, then plated out; $AS^{60}$: Serum-virus mixture incubated 1 hour before plating out. Illustrative of the dependence of neutralization on time *in vitro*. Discussion in the text.

to 5 minutes there is a considerable loss in effectiveness of neutralization, and if this time is further shortened as much as possible, there is additional loss in reaction, although, because of the time necessarily involved in the process of inoculation, it was impossible to show whether the reaction can be completely eliminated. The corresponding mixtures of normal serum and virus showed no essential difference in reaction regardless of whether the time of incubation was 1 hour or momentary.

This experiment shows that the reaction of immune serum with virus is a reaction that occurs *in vitro*, since it requires time *in vitro* for its full accomplishment. Such a finding is entirely consistent with the view that

the inhibitory effect of immune serum is a reaction affecting the virus, independent of any detrimental effect on the host plant. The fact that the time between 2 minutes or less and 1 hour has no appreciable effect on the normal serum reaction likewise indicates that the reaction of normal serum is a reaction that does not take place *in vitro,* a finding that is again consistent with the view that the reaction of normal serum is a reaction on the host plant, nor on the virus.

So far as has been observed, the reaction between immune serum and virus is largely accomplished during the first 10–15 minutes of incubation at room temperature. The degree of reaction after 1 hour's incubation at room temperature is not appreciably increased by increasing the incubation time to as much as 36 hours, including 2 hours at 37° C. On the other hand, as figure 7 shows, the reaction after 2–5 minutes is by no means complete.

### Specificity of the Neutralization Reaction

In order to study the neutralization of a virus by immune serum it is necessary to have some fairly accurate method for measuring the infectivity of virus samples. Tobacco mosaic is ideal for this purpose, because concentrated virus preparations produce as many as 1600 or more local lesions on the bean leaf and because such virus may be diluted as much as 1:10,000,000 without total loss of demonstrable infectivity. Thus it was inevitable that the greatest part of the work reported in the present paper with regard to the nature and properties of the reactions studied was carried out using tobacco-mosaic virus. Only two other of the viruses available have as yet yielded even partially to attempts at accurate quantitative methods, these two being tobacco ring spot and cucumber mosaic. Tobacco ring spot, if plated out on the cowpea, may give as many as 60 or more lesions per leaf, using undiluted expressed sap as inoculum, and is infectious to cowpea in dilutions up to 1:125, cucumber mosaic is much less infective, an average of 10 lesions per leaf with concentrated virus being exceptional, and dilutions infective to 1:100 rather rare. Often, because of physiological factors not yet understood, whole experiments must be rejected because of an infectivity too low to yield significant results. For these reasons the experiments described below have not yielded evidence as extensive and invariable as that from the study of tobacco mosaic; nevertheless, the experiments have been repeated a sufficient number of times to insure that the general trend of the results is significant.

That the neutralization reaction with the plant viruses be specific is not a necessary concomitant of its being a true serological reaction. With bacteriophage, for example, there is considerable disagreement as to the specificity of the neutralization test. Yet, with many of the animal viruses, the neutralization reaction is delicately specific, this specificity even extend-

ing to strain distinctions as in the case of the various strains of the foot-and-mouth-disease virus. It has, therefore, been of particular interest to test the specificity of the neutralization reaction with the three plant viruses available and with their various strains.

If a series of dilutions of tobacco-mosaic virus is treated with constant amounts of immune serum for tobacco mosaic there results an inactivation characterized by a sharply falling neutralization curve, as has been pointed out above. If at the same time the same virus dilutions are treated in the same way with the same concentrations of comparable antisera against cucumber mosaic or tobacco ring spot, these latter immune sera behave in no way different from normal serum with respect to the tobacco-mosaic virus. This situation is illustrated by the results of a typical experiment

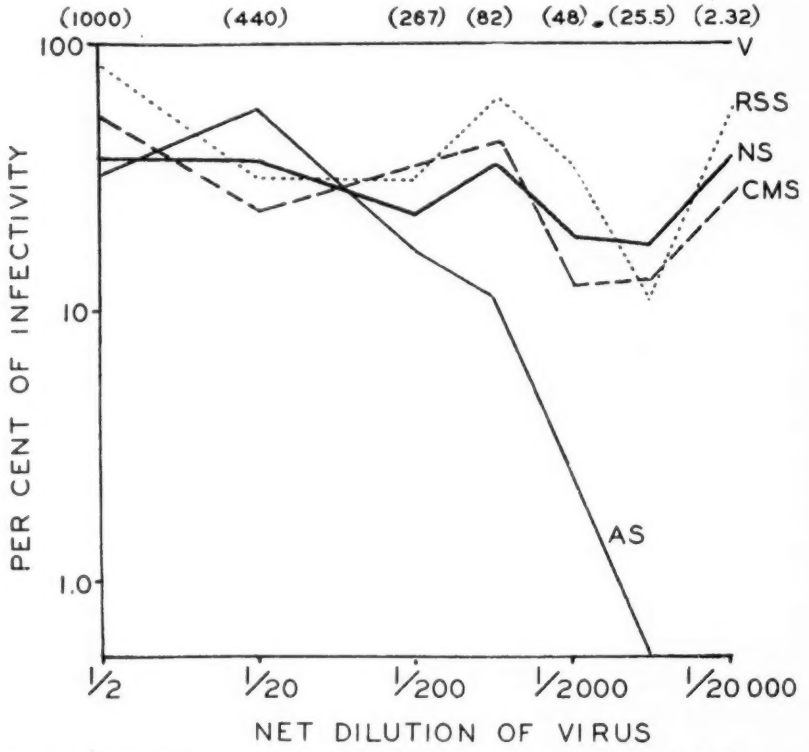


FIG. 8. Specificity of neutralization with tobacco-mosaic virus subjected to treatment with normal serum (NS), tobacco-ring-spot serum (RSS), cucumber mosaic serum (CMS), and anti-tobacco-mosaic serum (AS), all at dilutions of 1: 200 and all expressed in terms of serum-free control (V). Percentage of infectivity determined by lesion counts on bean. The same sera were used in figures 8–10, and these sera were all produced in similar manner from the injection of rabbits with expressed Turkish tobacco sap containing the viruses in question.

shown in figure 8, an experiment that has been several times repeated using different sera and virus preparations and always with essentially the same results.

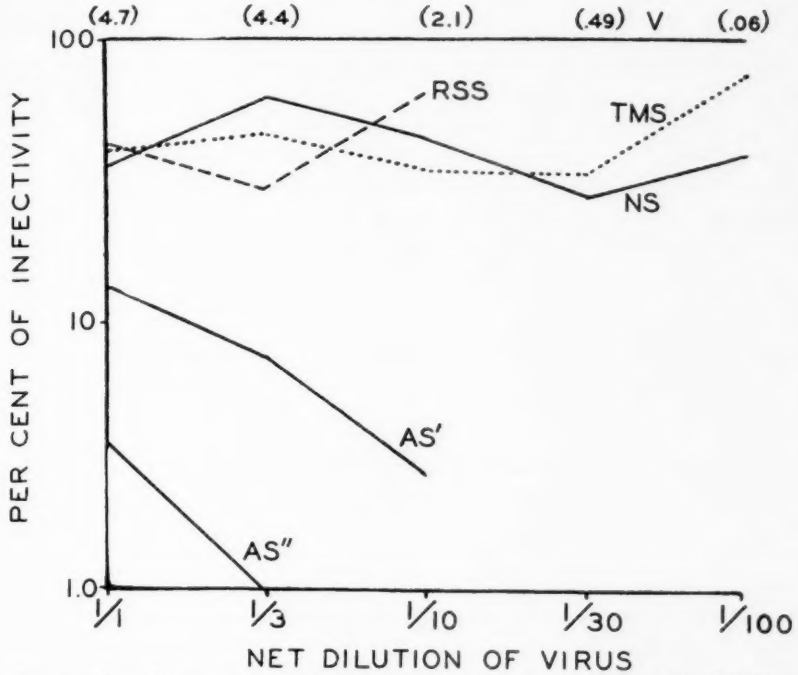


FIG. 9. Specificity of neutralization of cucumber-mosaic virus subjected to treatment with tobacco-mosaic serum (TMS), tobacco-ring-spot serum (RSS), and with two anti-cucumber-mosaic sera (AS′, AS″), all at dilutions of 1: 50 and all expressed in terms of infectivity of untreated virus control (V) at the same dilutions. Percentage of inhibition determined by infectivity counts on cowpea. Further discussion in the text.

If we now treat cucumber-mosaic virus with the same three sera, only the anti-cucumber-mosaic serum causes a falling curve, the other two sera behaving just as normal sera (Fig. 9). If finally we use tobacco-ring-spot virus, this virus becomes inactivated only by the anti-tobacco-ring-spot serum, not by the anti-cucumber-mosaic or anti-tobacco-mosaic sera (Fig. 10).

Thus, of the three virus types before us, tobacco-mosaic virus is inactivated only by anti-tobacco mosaic serum, cucumber-mosaic virus only by anti-cucumber mosaic serum, and tobacco-ring-spot virus only by anti-tobacco-ring-spot serum—the cross specificity is absolute, and the addition to any of these viruses of a heterologous antiserum exhibits no effect different from the host effect associated with all sera, normal or immune.

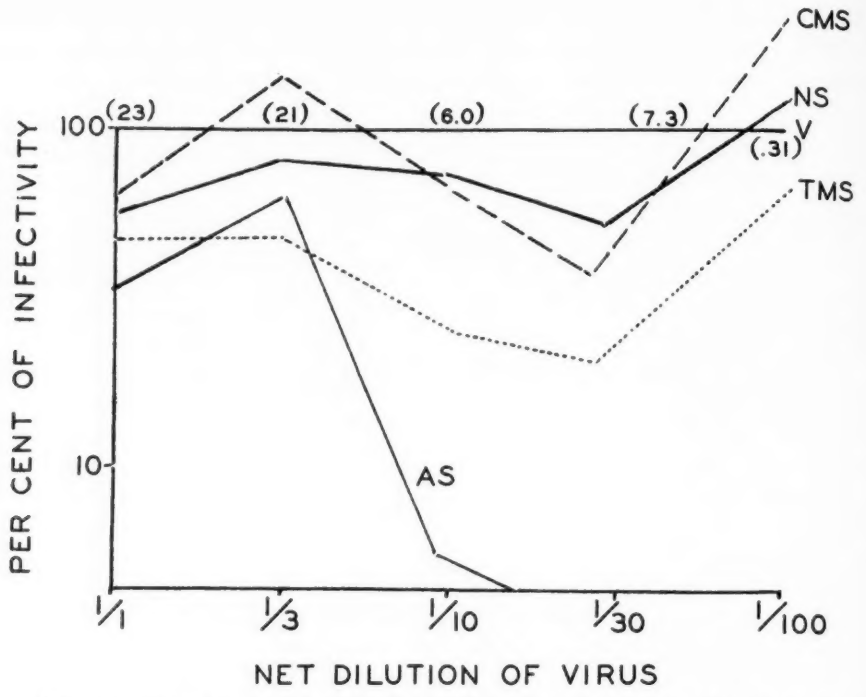


FIG. 10. Specificity of neutralization of tobacco-ring-spot virus subjected to treatment with tobacco-mosaic serum (TMS), cucumber-mosaic serum (CMS), anti-tobacco-ring-spot serum (AS), and normal serum (NS), all at dilutions of 1: 50 and all plotted in terms of the infectivity of the serum-free control (V). Inhibition measured by infectivity counts on cowpea. Discussion in the text.

Among the strains of any one of the three virus types studied, the evidence available up to the present indicates that such strains as have been tested are serologically very closely related. This subject will be mentioned only very briefly since the experiments involved are still being continued. Suffice it to say that with cucumber mosaic and tobacco mosaic no serological differences in strains of the same virus type have as yet been noted. With the apparently very different yellow and green strains of cucumber mosaic used, complete cross tests have not been performed as yet, but all of the data on cucumber mosaic given above have been obtained from tests of antigreen cucumber-mosaic serum with solutions of the yellow strain of virus. That a specific reaction does occur here evidences the close serological relationship of these two strains in spite of the very different symptoms produced.

With tobacco mosaic, cross neutralization tests using brilliant white, ordinary, and symptomless strains, have yielded no evidence as yet that

these remarkably different appearing strains possess any serological distinctness from one another. Figure 4 illustrates a typical experiment of this sort. The virus used was the white strain of tobacco mosaic "Jensen 108." It is seen that when treated with antiordinary or antiwhite tobacco mosaic serum the results are essentially the same in both cases, either antiserum neutralizing the white virus to approximately the same degree. The reciprocal test gave essentially the same results.

It is remarkable that certain yellow strains of tobacco mosaic and cucumber mosaic are at times indistinguishable on tobacco and capable of being confused by the connoisseur. Differences in host range and other properties have classified these virus strains in their present positions. The serological evidence entirely confirms that obtained from a study of host range and other properties in showing that the yellow strain of cucumber mosaic employed is closely related to the very different appearing green strains of cucumber mosaic, while the same yellow strain of cucumber mosaic is wholly unrelated to a superficially indistinguishable yellow strain of tobacco mosaic.

## SUMMARY

It is known from the work of earlier investigators that normal rabbit serum decreases the infectivity of tobacco-mosaic virus and that anti-tobacco-mosaic serum shows a greater inhibitory effect. This phenomenon has been further studied with the following results:

A technique is described which permits the differentiation of virus-inhibitory effects acting through decreasing the susceptibility of the host plant, and inhibitory effects due to inactivation of the viruses.

Using this technique it is shown that normal rabbit serum inhibits virus infectivity through decreasing the susceptibility of the host, not through an effect on the virus. This property is shared by other nonspecific proteinaceous substances, *e.g.*, healthy tobacco extract, ovalbumin, and milk.

Virus-immune serum, beside showing the same, non-specific host effect as normal serum, also exerts a specific neutralizing action on the virus used in immunization.

Neutralization of the virus of tobacco mosaic follows a law of multiple proportions: if the amount of virus be increased by a given percentage, the amount of immune serum added must be increased by the same percentage in order to produce the same inhibitory effect.

The host-inhibitory effect of normal serum is independent of time *in vitro* while neutralization of virus by immune serum requires time *in vitro* for its accomplishment.

There is an absolute cross specificity in the neutralization of the viruses

of tobacco mosaic, tobacco ring spot, and cucumber mosaic, such that each of these viruses is completely neutralized by its homologous serum, but not at all by the heterologous sera. As far as has been tested at present this specificity does not extend to virus strain distinctions.

The experiments show that the reaction in question is a specific, quantitative neutralization of virus by immune serum, such as has not heretofore been described for the plant viruses. The reaction has been used in the determination of the relationships of certain plant viruses and offers possibilities of further application in the study of the fundamental problems of plant virus research.

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# INVESTIGATIONS ON PHYSIOLOGIC SPECIALIZATION OF TILLETIA LAEVIS IN KANSAS[1]

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## INTRODUCTION

The bunt problem in the Great Plains has become very important during the last 8 years. Early records of wheat production in Kansas indicate that bunt, caused by *Tilletia laevis* Kühn, was comparatively uncommon and that from 1900 to 1917 very little seed wheat was treated for its control. During that period strains of Turkey wheat (Crimean) were commonly grown in Kansas. Since then new varieties of hard red winter wheats (Blackhull and Kanred) have been extensively grown and, while it is true that they are similar to the original Turkey, they are much more bunt-susceptible. Coincident with the growing of these new varieties, the bunt problem became one of tremendous importance in Kansas, where, in 1926, the loss caused by this disease was estimated at about $14,811,000 (13).

Whether the epiphytotics of bunt that have occurred in the Great Plains since 1925 are due more to changes in the varieties of wheat, or to variations in the fungus, *Tilletia laevis,* is speculative. It seems probable that both of these factors, as well as seasonal conditions and the wider distribution of smutty seed wheat, are involved.

## PHYSIOLOGIC FORMS OF BUNT AND THEIR SIGNIFICANCE

It has been known for several years that physiologic forms occur in bunt and other smuts (4, 12, 15, 17, 20). Recent investigations (1, 2, 6, 7, 8, 9) have shown that there are several physiologic forms of both *Tilletia tritici* (Bjerk.) Wint. and *T. laevis.* Reed (15) and Roemer (19) have proved that collections of bunt from various States in the United States and from Hungary, Egypt, Italy, Norway, New Zealand, Czechoslovakia, Austria, England, and Germany are composed of various physiologic forms of *T. tritici* and *T. laevis.* It also has been clearly demonstrated that there is a vast difference in the reaction of varieties of wheat to bunt collected from different localities. Certain varieties that at one time were regarded as resistant to bunt are no longer resistant (2). Flor (6) states that

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certain varieties maintained their resistance for a number of years, but are now being attacked with increased severity from year to year.

Studies on heterothallism and hybridization in the bunt fungi (6) show that the possibilities of the origination of new and more virulent forms of *Tilletia tritici* and *T. laevis* are unlimited. In the light of present knowledge mutations in the bunt organisms may also be expected. Certainly, the bunt organism must be regarded as more or less unstable in its pathogenicity when one considers that sporidial fusion (5) is necessary to the production of a smutted spikelet of wheat. Hybridization, therefore, frequently occurs and many of the chlamydospore collections used in infection studies in the field are essentially hybrids. Several investigators now consider chlamydospore collections as physiologic forms, although there are arguments to be raised against this opinion. This point of view, however, is followed in this paper.

Because of variations in the fungus, the search for resistance to bunt in winter and spring wheats has become not only greatly complicated, but it is now realized that in most cases a resistant variety, if secured, will be only a temporary attainment. In some of the tests for varietal resistance to bunt by artificial inoculation, only one or two collections of bunt have been used. These would not be representative of the bunt flora over a wide area. This criticism has been suggested by other workers (2, 4). In testing wheat varieties, selections, and hybrids for their reaction to bunt, the collections of smut used for inoculation should be secured from several counties in a State, or even from wheat-growing areas in several States. Whether state or even national boundaries should limit the source of inoculum will depend upon the area to which it is expected or hoped the new variety may be adapted. The reaction of a variety at one station to one form of bunt may be a very poor criterion of its behavior over a wider area.

The complexity of the situation may be realized when one considers that, theoretically, a single smutted head of wheat may contain not only both species of Tilletia but, also, several physiologic forms each of varying degrees of pathogenicity; that each chlamydospore represents a diploid phase of the fungus; and that sporidial fusion has taken place before a smut ball was produced.

There is, however, abundant evidence that varieties of commercial value can be produced that show marked resistance to bunt in the field, such as Ridit (3, p. 932), Albit, Oro, and the Turkey selections recently made at the Nebraska station (11). The ability of these varieties to remain resistant may be limited to a comparatively short time, however; in fact, some of them are now known to be susceptible to some forms of bunt. If a commercial variety, however, remains resistant for a period of 5 to 10

years, there is ample justification for all the work required to obtain such a variety, and investigators should realize the necessity of continuous work on this problem. There are numerous cases among the smut fungi (18) in which profound changes have occurred in the host-organism relationship. One needs only to consider what has taken place with Ridit, Oro, and Martin (2), and some of the "resistant" durum wheats and Marquis with respect to bunt (10), Kanota oats and other resistant Fulghum selections with respect to oat smut (15), and milo, feterita, and some of the other resistant sorghums in regard to kernel smut (14).

## PURPOSE OF EXPERIMENTS

The primary objective of these experiments was to prepare the way for the production of a desirable bunt-resistant hard winter wheat. In order to gain some knowledge of the bunt flora of Kansas, a number of collections of bunt have been made from several counties. These have been studied on a group of wheat varieties in order to separate the smut collections into what may be called physiologic forms. An attempt has been made to learn the distribution of these forms in Kansas. A study has been made also of certain of these forms on artificial media. Some inoculation experiments have shown that resistant varieties may act as media for building up more virulent physiologic forms of bunt. This process has been described by other investigators (21).

The studies here reported are not considered final; in fact, it is probable that other physiologic forms could be demonstrated if larger numbers of collections of bunt from all counties and regions of Kansas were made and if more varieties of wheat were used as differential hosts. It also is recognized that assigning certain bunt collections to a definite form number is not always possible. Furthermore, the form number assigned may in some cases be incorrect. The point that the writer wishes to emphasize is that distinct bunt strains of differing virulence exist in Kansas. A knowledge of their prevalence and distribution is of importance to those engaged in wheat improvement. Plant pathologists and plant breeders should not fail to give careful thought to this matter in making and carrying out plans for producing new varieties of commercial value. The grave mistake of distributing a new variety without knowing its reaction to the important diseases that may attack it has been made too frequently in the past. Aamodt (1) mentions the danger attending the introduction and growing of spring varieties of wheat such as Kota, Ceres, and Progress, even though they are resistant to stem rust. The bunt reaction of these varieties had not been determined prior to their introduction. In this connection he says, "these new varieties of wheat then, together with the durum wheats, have greatly aggravated the bunt problem, not only because of the inability

of the varieties to withstand attacks by the bunt organism, but because they have been a medium through which the pathogene has become more thoroughly and widely distributed."

## METHODS AND MATERIALS

Bunt collections were made in Kansas in 1928 to 1931, inclusive, on susceptible commercial varieties of wheat, such as Kanred, Blackhull, and Turkey. All these collections of bunt proved to be *Tilletia laevis*. The standard varieties of wheat used in these physiologic-form studies are winter wheats, planted in October of one year and harvested the following June.

The collections of smut were carefully prepared and each was handled as though it might be a distinct physiologic form or aggregate of forms. Generally, 2 sowings of the complete list of varieties were made from inoculated seed on 2 dates. One planting was made late enough in the fall to coincide with optimum conditions for infection. The seed of the standard varieties was disinfected before inoculating with the bunt spores. The ordinary methods for applying maximum spore loads were used, care being taken to insure that each variety received approximately the same amount of inoculum. The bunt nursery consisted of 5-foot rows planted a foot apart. The plantings in the bunt nursery for a given seeding date were all made simultaneously. During the 4 years in which these studies were made, the soil temperature range for a week following planting was 35° to 60° F. This range proved satisfactory and heavy infections resulted. When harvested each row was cut separately, tied, and labeled. Each head was examined for bunt. Percentage of bunt was determined on the basis of counts of smutted and normal heads. The total number of wheat heads per row ranged from 200 to 475.

The standard varieties used in these studies were selected from a list of many that had been reported to have differential reactions. Of the 12 varieties used, only 6 were finally selected as physiologic-form differentials. The standard varieties used were Banner Berkeley (C.I. 7362), Hussar (C.I. 4843), Oro (C.I. 8220), Ridit (C.I. 6703), Turkey (C.I. 1558A), Martin (C.I. 4463), White Odessa (C.I. 4655), Kooperatorka (C.I. 8861), Turkey × Bearded (Minnesota No. 48) (C.I. 8243), Yogo (Minturki × Beloglina × Buffum) (C.I. 8033), Regal (C.I. 7364), and Kanred (C.I. 5146). Of these the first 6 were used as differentials.

## SOIL INFESTATION OF BUNT

One of the reasons why bunt has not become a more serious limiting factor in wheat production in Kansas is that soil infestation by bunt spores does not occur. There has been no positive evidence in the past 20 years

that *Tilletia laevis* survives in the soils of this State. With the introduction and general use of the combine harvester in Kansas, it seemed possible that soils might become heavily contaminated with spores, especially in certain years when bunt was very prevalent. Apparently, environmental conditions are such that chlamydospores have become inactive by seeding time, when infection normally would occur. For this reason seed wheat that is properly treated is reasonably certain of producing a smut-free crop in Kansas.

## BUNT-COLLECTING CENTERS IN KANSAS

The upper map (Fig. 1) shows the bunt-collecting centers in Kansas and the years when the collections were made. For the most part, these collec-

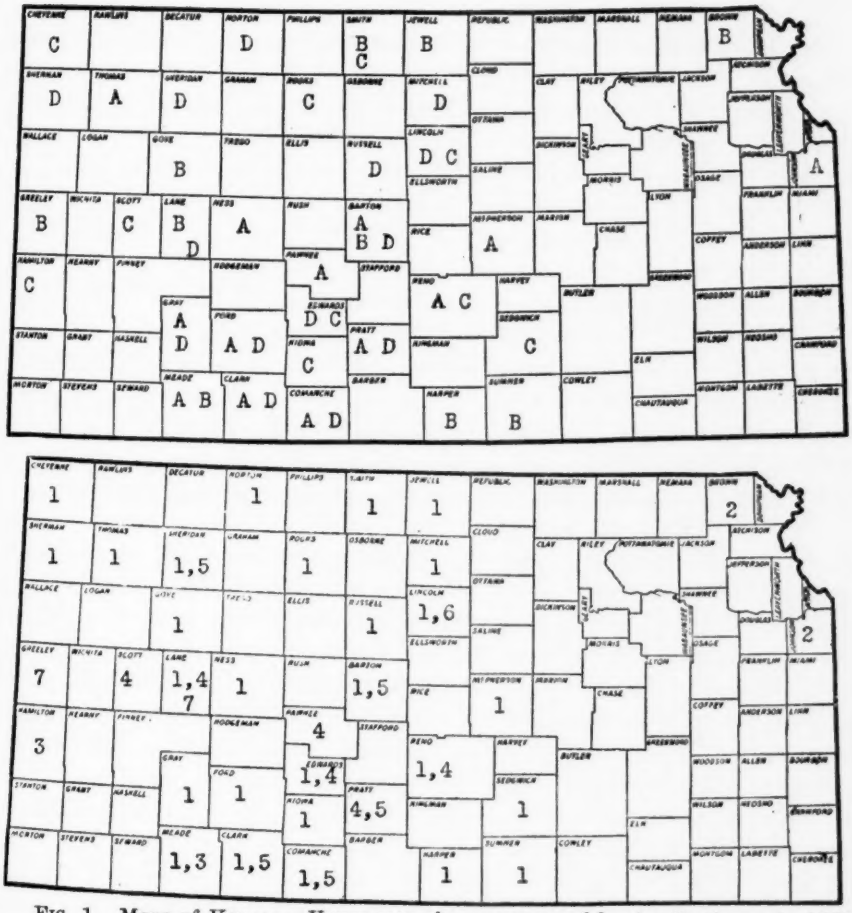

FIG. 1. Maps of Kansas. Upper map shows sources of bunt collections: A, 1928; B, 1929; C, 1930; D, 1931. Lower map shows distribution of physiologic forms of bunt.

tions were made in the principal winter-wheat counties of central and western Kansas. The varieties listed in the tables are arranged from left to right in the same order they were sown in the nursery. The reaction of each variety to the various collections of bunt, expressed in percentage of bunted heads, is given in the column under each variety.

In table 1 the county where the collection of bunt was made, the collection number, and the year are given. Since Kanred proved to be very susceptible to all bunt collections, the bunt present on this variety was saved each year as inoculum for all varieties in the next year's test. The results are listed separately for the various varieties tested. A total of 64 collections of bunt from widely scattered areas in the State were used in the course of these studies. In general, the reactions from year to year for the same collection were very similar. It is evident, however, that some collections of bunt did not infect the differential varieties to the same extent each year. This is to be expected, since a field collection may contain several forms all of which may vary in amount in the succeeding years.

There are distinct differences in pathogenicity to be noted in several of the bunt collections. Banner Berkeley has been recognized as a resistant variety, and, for the most part, it is resistant to the bunt collections made in Kansas. This is not true, however, for such collections as Brown County No. 17, Greeley County No. 19, Johnson County No. 8, and Lane County No. 53, since Banner Berkeley had 59.5, 19.4, 59.8, and 20.3 per cent of bunt, respectively, from these collections. Hussar, which also is usually highly resistant, produced 14.1 per cent infection in the case of Meade County No. 23 collection. Martin was highly resistant to most of the bunt collections used in these studies, but several collections were decidedly virulent on this variety, as, for example, Brown County No. 17, Johnson County No. 8, and Meade County No. 23, which gave 39.7, 39.6, and 14.4 per cent of bunt, respectively. Oro was highly resistant to all collections except Lincoln County No. 56, which produced 8.7 per cent infection. Ridit was markedly resistant to all collections except Barton County No. 2, Clark County No. 3, and Meade County No. 23, where moderate infections of 11.2, 10.4, and 8.9 per cent, respectively, were produced. Turkey Sel. No. 1558A was one of the most consistently resistant varieties subjected to the Kansas bunt collections. As will be shown later, however, such infections on the Turkey selection as 8.9 per cent smut in Edwards County No. 49 and 14.4 per cent in Pratt County No. 14 are significant differences.

There also are marked differences in the reaction of White Odessa, Kooperatorka, Turkey × Bearded, Yogo, and Regal to the various bunt collections. These varieties were not used in constructing the key for the separation of the physiologic forms; however, some of these wheats could have been so used. In contrast to the resistant varieties discussed, it is of

TABLE 1.—*Reaction of a selected group of wheat varieties to collections of bunt, Tilletia laevis, made in various counties in Kansas, and tested at Manhattan during the period, 1929 to 1932, inclusive*

| Source of collection | Collection No. | Year | | Percentage of heads bunted | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Collected | Grown | Banner Berkeley | Hussar | Oro | Ridit | Turkey 1558A | Martin | White Odessa | Kooperatorka | Turkey × Bearded | Yogo | Regal | Kanred |
| *County* | | | | | | | | | | | | | | | |
| Barton | 1 | 1928 | 1929 | 0.0 | 0.0 | 3.1 | 5.7 | 0.2 | 0.0 | 0.2 | 0.4 | 0.0 | 2.8 | 0.4 | 61.9 |
| " | 2 | " | " | 0.0 | 0.0 | 0.6 | 11.2 | 0.0 | 0.0 | 0.0 | 1.5 | 0.0 | 2.2 | 2.5 | 59.4 |
| " | 16 | 1929 | 1930 | 0.0 | 0.0 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 1.0 | 2.5 | 0.4 | 0.0 | 44.5 |
| " | " | " | 1931 | 0.0 | 0.0 | 0.0 | 1.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.8 | 0.0 | 47.1 |
| " | " | " | 1932 | 0.7 | 0.0 | 1.4 | 0.0 | 2.2 | 0.0 | 0.0 | 0.0 | 0.0 | 2.0 | 0.0 | 22.1 |
| " | 37 | 1931 | " | 0.0 | 0.0 | 0.7 | 0.4 | 1.2 | 0.0 | 0.0 | 0.8 | 0.0 | 2.1 | 0.0 | 27.5 |
| " | 38 | " | " | 0.0 | 0.0 | 0.4 | 1.1 | 2.4 | 0.0 | 0.0 | 0.7 | 0.9 | 0.6 | 0.2 | 12.7 |
| " | 39 | " | " | 1.2 | 0.0 | 0.0 | 0.4 | 0.4 | 0.0 | 0.0 | 0.4 | 0.0 | 2.8 | 0.0 | 61.7 |
| Brown | 17 | 1929 | 1930 | 23.1 | 0.0 | 0.0 | 0.0 | 1.0 | 2.2 | 2.1 | 4.3 | 1.0 | 0.0 | 2.5 | 25.3 |
| " | " | " | 1931 | 59.5 | 0.0 | 0.0 | 0.0 | 0.9 | 39.7 | 27.5 | 18.0 | 0.0 | 0.0 | 7.4 | 18.4 |
| " | " | " | 1932 | ..... | 0.0 | 0.0 | 0.0 | 2.3 | 1.9 | 4.8 | 3.8 | 1.5 | 0.7 | 8.0 | 8.4 |
| Cheyenne | 26 | 1930 | 1931 | 0.0 | 0.0 | 0.0 | 0.0 | 3.9 | 0.0 | 0.0 | 0.0 | 0.0 | 2.2 | 0.0 | 48.4 |
| " | " | " | 1932 | 0.0 | 0.0 | 0.0 | 0.0 | 0.6 | 0.0 | 0.0 | 0.0 | 1.4 | 3.7 | 0.0 | 44.0 |
| Clark | 3 | 1928 | 1929 | 0.0 | 0.0 | 2.4 | 10.4 | 0.0 | 0.0 | 0.3 | 2.3 | 0.0 | 4.5 | 0.2 | 41.6 |
| " | 40 | 1931 | 1932 | 0.0 | 0.0 | 0.3 | 0.6 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 3.9 | 0.0 | 30.3 |
| " | 41 | " | " | 0.0 | 0.6 | 0.0 | 1.9 | 1.6 | 0.0 | 0.0 | 0.0 | 0.5 | 1.7 | 0.0 | 33.8 |
| Comanche | 4 | 1928 | 1929 | 0.0 | 0.0 | 0.6 | 5.1 | 0.8 | 0.0 | 0.0 | 5.6 | 0.0 | 1.3 | 2.1 | 60.4 |
| " | 42 | 1931 | 1932 | 2.1 | 1.1 | 0.6 | 1.3 | 1.6 | 0.0 | 0.0 | 0.3 | 0.0 | 0.4 | 0.0 | 20.9 |
| " | 43 | " | " | 1.4 | 0.4 | 0.3 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 1.7 | 0.0 | 23.9 |
| Edwards | 27 | 1930 | 1931 | 5.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 | 0.0 | 0.0 | 0.5 | 0.0 | 0.0 | 55.6 |
| " | " | " | 1932 | 0.4 | 1.1 | 1.1 | 1.0 | 1.2 | 0.0 | 0.0 | 2.3 | 0.0 | 0.7 | 0.2 | 37.4 |
| " | 46 | 1931 | " | 5.7 | 0.4 | 0.0 | 0.5 | 0.9 | 0.4 | 1.5 | 2.4 | 0.7 | 0.0 | 0.5 | 31.8 |
| " | 47 | " | " | 0.0 | 0.8 | 0.3 | 3.3 | 0.3 | 0.0 | 0.0 | 2.5 | 0.7 | 3.6 | 0.0 | 48.3 |
| " | 48 | " | " | 0.0 | 0.0 | 0.6 | ..... | 3.0 | 0.0 | 0.0 | 1.1 | 0.0 | 8.5 | 0.3 | 64.4 |
| " | 49 | " | " | ..... | 1.8 | 0.7 | 1.9 | 8.9 | 0.0 | 0.5 | 5.5 | 0.0 | 3.4 | 0.0 | 61.0 |
| Ford | 5 | 1928 | 1929 | 0.0 | 0.0 | 3.0 | 3.6 | 0.8 | 0.0 | 0.0 | 1.4 | 0.0 | 0.8 | 1.8 | 73.3 |
| " | 44 | 1931 | 1932 | 1.5 | 0.0 | 3.3 | 1.3 | 1.7 | 0.0 | 1.6 | 0.0 | 1.3 | 2.5 | 0.0 | 42.9 |
| " | 45 | " | " | 0.0 | 0.0 | 0.3 | 0.4 | 0.3 | 0.0 | 0.0 | 3.0 | 0.9 | 2.3 | 0.0 | 16.3 |
| Gove | 18 | 1929 | 1930 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.9 | 1.0 | 2.5 | 0.6 | 0.0 | 31.6 |
| " | " | " | 1931 | 0.0 | 0.0 | 0.0 | 3.7 | 1.8 | 0.0 | 0.0 | 0.0 | 1.4 | 0.0 | 0.0 | 50.0 |
| " | " | " | 1932 | 0.0 | 1.1 | 2.0 | 3.1 | 1.1 | 0.0 | 0.0 | 1.1 | 0.0 | 7.9 | 0.0 | 51.8 |
| Gray | 6 | 1928 | 1929 | 0.0 | 0.0 | 1.2 | 1.4 | 0.4 | 0.0 | 0.0 | 1.8 | 0.0 | 0.8 | 0.0 | 60.3 |
| " | 7 | " | " | 0.0 | 0.9 | 3.0 | 2.4 | 0.0 | 0.0 | 0.0 | 2.7 | 0.0 | 0.0 | 0.7 | 68.3 |
| " | 50 | 1931 | 1932 | ..... | 0.0 | 3.4 | 1.5 | 1.0 | 0.0 | 0.0 | 0.6 | 0.3 | 1.2 | 1.0 | 35.4 |
| Greeley | 19 | 1929 | 1930 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 | 22.9 |
| " | " | " | 1931 | 19.4 | 0.0 | 1.0 | 2.0 | 0.0 | 0.0 | 7.1 | 2.1 | 1.4 | 1.2 | 0.8 | 54.3 |
| " | " | " | 1932 | 13.3 | 0.5 | 0.0 | 0.8 | 4.2 | 3.4 | 0.9 | 1.7 | 0.0 | 4.1 | 0.3 | 39.9 |
| Hamilton | 28 | 1930 | 1931 | 2.6 | 7.5 | 0.0 | 0.0 | 2.4 | 2.5 | 7.1 | 1.6 | 1.6 | 1.6 | 0.4 | 52.2 |
| " | " | " | 1932 | 5.4 | 1.7 | ..... | 0.0 | 0.3 | 1.3 | 0.0 | 0.3 | 0.0 | 2.8 | 0.3 | 15.4 |
| Harper | 20 | 1929 | 1930 | 0.0 | 0.0 | 0.0 | 0.0 | 4.2 | 1.0 | 0.5 | 0.7 | 0.6 | 1.1 | 0.0 | 34.3 |
| " | " | " | 1931 | 4.9 | 0.0 | 1.0 | 1.9 | 0.0 | 3.9 | 3.2 | 1.9 | 0.6 | 2.2 | 0.7 | 36.7 |
| " | " | " | 1932 | 5.4 | 0.0 | 0.3 | 0.5 | 0.6 | 0.0 | 0.0 | 2.9 | 0.5 | 6.2 | 0.0 | 71.4 |
| Jewell | 21 | " | 1930 | 0.0 | 0.0 | 0.4 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 0.0 | 26.9 |
| " | " | " | 1931 | 0.0 | 0.0 | 0.0 | 1.4 | 3.2 | 0.0 | 0.0 | 0.0 | 0.6 | 0.0 | 0.0 | 44.0 |
| " | " | " | 1932 | 0.0 | 0.0 | 0.5 | 4.0 | 1.2 | 0.0 | 0.3 | 0.3 | 0.2 | 0.9 | 0.0 | 51.9 |
| Johnson | 8 | 1928 | 1929 | 34.4 | 0.0 | 0.7 | 0.0 | 0.6 | 5.9 | 6.9 | 15.3 | 0.0 | 1.4 | 5.5 | 49.9 |
| " | " | " | 1930 | 7.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.9 | 1.0 | 4.5 | 0.0 | 0.0 | 2.4 | 11.4 |
| " | " | " | 1931 | 59.8 | 0.9 | 0.0 | 0.0 | 5.9 | 39.6 | 18.0 | 25.1 | 0.9 | 1.8 | 6.3 | 35.9 |

TABLE 1.—*(Continued)*

| Source of collection | Collection No. | Year | | Percentage of heads bunted | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Collected | Grown | Banner Berkeley | Hussar | Oro | Ridit | Turkey 1558A | Martin | White Odessa | Kooperatorka | Turkey × Bearded | Yogo | Regal | Kanred |
| *County* | | | | | | | | | | | | | | | |
| Johnson | 8 | 1928 | 1932 | 31.0 | 1.1 | 0.8 | 0.7 | 0.6 | 5.6 | 9.7 | 24.5 | 1.1 | 0.3 | 1.9 | 45.9 |
| Kiowa | 29 | 1930 | 1931 | 3.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 1.9 | 0.0 | 0.0 | 0.0 | 61.4 |
| " | " | " | 1932 | 2.1 | 0.8 | 1.1 | 0.4 | 2.4 | 0.3 | 0.4 | 0.6 | 0.0 | 6.5 | 0.0 | 44.8 |
| Lane | 22 | 1929 | 1930 | 0.0 | 0.0 | 0.0 | 0.5 | 4.1 | 0.0 | 0.0 | 1.6 | 0.0 | 0.0 | 0.0 | 19.1 |
| " | " | " | 1931 | 0.0 | 0.0 | 0.0 | 0.9 | 5.8 | 0.0 | 0.0 | 2.4 | 0.7 | 0.8 | 0.0 | 54.4 |
| " | " | " | 1932 | 0.0 | 1.7 | 0.3 | 1.5 | 1.4 | 0.0 | 0.6 | 0.9 | 1.2 | 2.8 | 0.0 | 41.6 |
| " | 51 | 1931 | " | 0.5 | 1.0 | 0.0 | 0.9 | 1.3 | 0.0 | 0.0 | 1.9 | 1.0 | 3.5 | 0.4 | 60.8 |
| " | 52 | " | " | 0.4 | 0.0 | 0.0 | 1.3 | ..... | 0.0 | ..... | 0.0 | ..... | 1.4 | 0.0 | 33.7 |
| " | 53 | " | " | 20.3 | 3.6 | 0.3 | 1.4 | 1.8 | 1.4 | 1.8 | 1.4 | 0.0 | 0.7 | 0.3 | 43.1 |
| " | 54 | " | " | 0.8 | ..... | 0.7 | 0.4 | 0.6 | 0.0 | 0.0 | 1.0 | 1.3 | 6.8 | 0.0 | 65.8 |
| Lincoln | 30 | 1930 | 1931 | 0.0 | 0.0 | 0.0 | 2.2 | 0.0 | 0.0 | 0.0 | 3.9 | 0.0 | 2.7 | 0.0 | 67.8 |
| " | " | " | 1932 | 0.0 | 0.7 | 0.7 | 1.3 | 0.6 | 0.0 | 0.0 | 0.3 | 0.5 | 7.2 | 0.0 | 53.1 |
| " | 55 | 1931 | " | 0.0 | 0.0 | 0.0 | 2.1 | 3.0 | 0.0 | 0.0 | 1.4 | 0.0 | 3.5 | 0.0 | 68.2 |
| " | 56 | " | " | 6.3 | 2.6 | 8.7 | 2.1 | 3.6 | 1.0 | 0.4 | 1.0 | 1.2 | 7.4 | ..... | 73.2 |
| McPherson | 9 | 1928 | 1929 | 0.0 | 0.0 | 3.1 | 0.0 | 0.6 | 0.0 | 0.0 | 1.8 | 0.0 | 5.9 | 1.2 | 85.5 |
| Meade | 10 | " | " | 0.0 | 0.0 | 1.8 | 1.5 | 0.2 | 0.0 | 0.0 | 1.5 | 0.0 | 3.4 | 2.3 | 77.8 |
| " | 23 | 1929 | 1930 | 0.0 | 7.4 | 0.0 | 0.6 | 5.1 | 1.7 | 1.1 | 0.0 | 0.0 | 3.4 | 0.0 | 45.1 |
| " | " | " | 1931 | 6.0 | 14.1 | 0.0 | 8.9 | 5.2 | 14.4 | 15.0 | 2.8 | 0.0 | 1.1 | 0.0 | 56.1 |
| " | " | " | 1932 | 0.0 | 0.0 | 0.3 | 1.7 | 0.5 | 0.4 | 0.0 | 0.0 | 0.0 | 2.2 | 0.0 | 61.8 |
| Mitchell | 57 | 1931 | " | 5.3 | 0.0 | 1.5 | 1.1 | 0.3 | 0.0 | 0.4 | 0.7 | 0.0 | 1.1 | 0.0 | 39.5 |
| Ness | 11 | 1928 | 1929 | 0.2 | 0.0 | 0.0 | 0.0 | 1.4 | 0.0 | 2.2 | 6.5 | 0.2 | 2.6 | 3.7 | 72.1 |
| Norton | 58 | 1931 | 1932 | 1.9 | 0.5 | 1.5 | 1.6 | 1.4 | 0.0 | 0.0 | 1.2 | 0.0 | 1.3 | 0.0 | 46.5 |
| Pawnee | 12 | 1928 | 1929 | 0.2 | 0.0 | 0.0 | 0.5 | 6.2 | 0.0 | 0.0 | 0.6 | 0.8 | 2.8 | 0.7 | 82.3 |
| Pratt | 14 | " | " | 0.0 | 0.0 | 0.0 | 0.0 | 14.4 | 0.0 | 0.0 | 1.6 | 0.0 | 0.0 | 0.0 | 53.4 |
| " | 59 | 1931 | 1932 | 0.0 | 2.4 | 0.0 | 5.5 | 0.3 | 0.5 | 0.0 | 0.3 | 0.0 | 2.2 | 0.0 | 44.4 |
| Reno | 13 | 1928 | 1929 | 0.0 | 0.0 | 0.9 | 0.0 | 6.6 | 0.0 | 0.0 | 0.0 | 0.0 | 3.0 | 0.0 | 60.5 |
| " | 31 | 1930 | 1931 | 0.0 | 1.1 | 0.0 | 0.0 | 2.8 | 0.0 | 0.0 | 0.0 | 0.0 | 1.5 | 0.0 | 62.7 |
| " | " | " | 1932 | 1.9 | 0.0 | 0.0 | 1.7 | 2.0 | 0.0 | 0.0 | 0.8 | 0.7 | 1.4 | 0.0 | 34.1 |
| Rooks | 32 | " | 1931 | 0.0 | 0.0 | 0.0 | 1.3 | 0.0 | 0.0 | 0.0 | 2.3 | 1.3 | 1.8 | 0.0 | 66.5 |
| " | " | " | 1932 | 0.5 | 3.1 | 0.5 | 0.5 | 0.6 | 0.0 | 0.0 | 2.2 | 0.0 | 3.3 | 0.7 | 40.6 |
| Russell | 64 | 1931 | " | ..... | 1.4 | 1.1 | 0.5 | 0.0 | 0.5 | 0.0 | 1.8 | 0.3 | 1.0 | 0.0 | 50.9 |
| Scott | 36 | 1930 | 1931 | 6.4 | 1.4 | 0.0 | 0.0 | 7.3 | 0.0 | 0.0 | 3.9 | 0.0 | 0.0 | 1.6 | 70.0 |
| " | " | " | 1932 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.3 | 1.0 | 0.0 | 30.8 |
| Sedgwick | 33 | " | 1931 | 0.0 | 0.0 | 0.0 | 0.0 | 3.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 60.9 |
| " | " | " | 1932 | 3.4 | 0.3 | 0.7 | 0.0 | 0.8 | 0.4 | 0.0 | 0.9 | 0.0 | 3.1 | 0.0 | 33.8 |
| Sheridan | 60 | 1931 | " | 0.0 | 0.4 | 0.3 | 0.9 | 1.7 | 0.0 | 0.0 | 1.0 | 0.7 | 1.9 | 0.0 | 39.7 |
| " | 61 | " | " | 0.0 | 0.0 | 1.2 | 5.2 | 0.0 | 0.0 | 0.0 | 1.0 | 1.3 | 2.7 | 0.0 | 33.8 |
| Sherman | 62 | " | " | 1.1 | 1.1 | 0.0 | 1.4 | 2.5 | 0.9 | 0.0 | 1.5 | 0.0 | 2.8 | 1.5 | 29.9 |
| " | 63 | " | " | 2.7 | 0.0 | 0.0 | 1.5 | 2.0 | 0.0 | 0.0 | 0.3 | 0.3 | 0.0 | 0.0 | 23.6 |
| Smith | 24 | 1929 | 1930 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.0 | 0.0 | 1.8 | 0.0 | 0.0 | 0.0 | 33.6 |
| " | " | " | 1931 | 1.2 | 0.0 | 0.0 | 1.2 | 2.3 | 0.0 | 0.0 | 3.1 | 0.6 | 1.9 | 0.0 | 51.5 |
| " | 34 | 1930 | " | 2.4 | 0.0 | 0.0 | 3.5 | 0.0 | 0.0 | 0.0 | 1.5 | 0.6 | 0.8 | 0.0 | 73.2 |
| " | 35 | " | " | 0.0 | 0.0 | 0.0 | 1.2 | 0.0 | 0.0 | 0.0 | 3.9 | 1.4 | 1.3 | 0.0 | 44.0 |
| " | " | " | 1932 | 0.0 | 0.0 | 0.3 | 0.7 | 1.9 | 0.0 | 0.0 | 0.0 | 0.6 | 0.4 | 0.0 | 56.7 |
| Sumner | 25 | 1929 | 1930 | 0.0 | 2.2 | 0.0 | 0.0 | 2.1 | 0.0 | 0.0 | 0.8 | 0.0 | 0.0 | 0.0 | 23.8 |
| " | " | " | 1931 | 0.0 | 0.0 | 0.0 | 3.3 | 1.6 | 0.0 | 0.0 | 4.0 | 0.0 | 0.0 | 0.0 | 56.3 |
| " | " | " | 1932 | 0.0 | 0.0 | 1.4 | 0.0 | 0.6 | 0.0 | 0.0 | 0.0 | 1.1 | 0.3 | 0.3 | 23.0 |
| Thomas | 15 | 1928 | 1929 | 4.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 2.1 | 1.4 | 41.4 |

interest to note the consistent reaction of Kanred, a commercial variety of considerable importance, which is highly susceptible to every collection made.

One may raise the question whether a bunt percentage, such as 7.4 in the case of Meade County No. 23 in a variety like Hussar is sufficiently high to differentiate this collection as a distinct form of the bunt organism. This variety has zero or trace to 14+ per cent of bunt from all other collections. The fact that Meade County No. 23 collection is a distinct form with high infection potentialities is shown in tables 3 and 4. When the variety Hussar was successively inoculated with this collection of bunt for 3 years, the resulting infections were 7.4 per cent in 1930, 47.2 per cent in 1931, and 16.6 per cent bunt in 1932. The striking reaction of this collection of smut when used to inoculate the differential varieties in 1932, after it had been increased on Hussar for two years, is shown in table 4. This shows plainly how a new form may be separated and increased on a variety that is generally regarded as highly resistant. This process was observed in other instances during the course of these investigations. In a few cases, however, such apparent increases proved to be mere fluctuations. The subject of reinoculations is more fully discussed on page 1216.

A summary of studies of the bunt collections is given in table 2 where the collections are grouped into physiologic forms according to their reaction on the differential varieties. Where a collection of bunt has been studied for 2 or more years, an average of the smut percentages for each variety has been made.

The physiologic-form numbers that have been assigned in this paper have no direct connection with the numbers assigned by other investigators of the bunt problem, since the differential varieties used are not necessarily the same. The writer realizes that some of the physiologic forms here recognized probably are questionable because the percentages of bunt are rather low, but since it was amply demonstrated that they differed from known forms, numbers have been assigned to readily distinguish them. It became apparent in these studies that a definite classification of all collections of bunt is not possible, but an attempt has been made to group the collections that have similar pathogenic tendencies on the standard varieties of wheat. In studies of this kind there always is a question in regard to form differentiation; *i.e.*, whether or not some physiologic forms have not been separated on too narrow a margin. This may be the case in some instances in these studies. However, if the data are studied statistically over a period of years, it is very probable that they will be found to be significant.

It is well known that bunt percentages differ from year to year because of environmental conditions. This was demonstrated in these studies by

TABLE 2.—*The average percentage of bunt obtained on a selected group of wheat varieties, and the physiologic form numbers assigned to the bunt collections, Manhattan, Kansas*

| Source of smut | Collection No. | Year collected | Number of years tested | Average percentage of smutted heads | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Banner Berkeley | Hussar | Oro | Ridit | Turkey Sel. 1558A | Martin | White Odessa | Kooperatorka | Turkey × Bearded | Yogo | Regal | Kanred |
| *County* | | | | *Physiologic Form 1* | | | | | | | | | | | |
| Barton | 16 | 1929 | 3 | 0.2 | 0.0 | 0.5 | 0.8 | 0.7 | 0.0 | 0.0 | 0.4 | 0.8 | 1.1 | 0.0 | 37.9 |
| " | 37 | 1931 | 1 | 0.0 | 0.0 | 0.7 | 0.4 | 1.2 | 0.0 | 0.0 | 0.8 | 0.0 | 2.1 | 0.0 | 27.5 |
| " | 38 | " | 1 | 0.0 | 0.0 | 0.4 | 1.1 | 2.4 | 0.0 | 0.0 | 0.7 | 0.9 | 0.6 | 0.2 | 12.7 |
| " | 39 | " | 1 | 1.2 | 0.0 | 0.0 | 0.4 | 0.4 | 0.0 | 0.0 | 0.4 | 0.0 | 2.8 | 0.0 | 61.7 |
| Cheyenne | 26 | 1930 | 2 | 0.0 | 0.0 | 0.0 | 0.0 | 2.3 | 0.0 | 0.0 | 0.0 | 0.7 | 3.0 | 0.0 | 46.2 |
| Clark | 40 | 1931 | 1 | 0.0 | 0.0 | 0.3 | 0.6 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 3.9 | 0.0 | 30.3 |
| " | 41 | " | 1 | 0.0 | 0.6 | 0.0 | 1.9 | 1.6 | 0.0 | 0.0 | 0.0 | 0.5 | 1.7 | 0.0 | 33.8 |
| Comanche | 42 | 1931 | 1 | 2.1 | 1.1 | 0.6 | 1.3 | 1.6 | 0.0 | 0.0 | 0.3 | 0.0 | 0.4 | 0.0 | 20.9 |
| " | 43 | " | 1 | 1.4 | 0.4 | 0.3 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 1.7 | 0.0 | 23.9 |
| Edwards | 27 | 1930 | 2 | 2.7 | 0.5 | 0.5 | 0.5 | 0.6 | 0.2 | 0.0 | 1.1 | 0.2 | 0.4 | 0.1 | 46.5 |
| " | 46 | 1931 | 1 | 5.7 | 0.4 | 0.0 | 0.5 | 0.9 | 0.4 | 1.5 | 2.4 | 0.7 | 0.0 | 0.5 | 31.8 |
| " | 47 | " | 1 | 0.0 | 0.8 | 0.3 | 3.3 | 0.2 | 0.0 | 0.0 | 2.5 | 0.7 | 3.6 | 0.0 | 48.3 |
| " | 48 | " | 1 | 0.0 | 0.0 | 0.6 | ..... | 3.0 | 0.0 | 0.0 | 1.1 | 0.0 | 8.5 | 0.3 | 64.4 |
| Ford | 44 | 1931 | 1 | 1.5 | 0.0 | 3.3 | 1.3 | 1.7 | 0.0 | 1.6 | 0.0 | 1.3 | 2.5 | 0.0 | 42.9 |
| " | 45 | " | 1 | 0.0 | 0.0 | 0.3 | 0.4 | 0.3 | 0.0 | 0.0 | 3.0 | 0.9 | 2.3 | 0.0 | 16.3 |
| " | 5 | 1928[a] | 1 | 0.0 | 0.0 | 3.0 | 3.6 | 0.8 | 0.0 | 0.0 | 1.4 | 0.0 | 0.8 | 1.8 | 73.3 |
| Gove | 18 | 1929 | 3 | 0.0 | 0.4 | 0.7 | 2.3 | 1.0 | 0.0 | 0.3 | 0.7 | 1.3 | 2.9 | 0.0 | 44.4 |
| Gray | 6 | 1928[a] | 1 | 0.0 | 0.0 | 1.2 | 1.4 | 0.4 | 0.0 | 0.0 | 1.8 | 0.0 | 0.8 | 0.0 | 60.3 |
| " | 7 | " [a] | 1 | 0.0 | 0.9 | 3.0 | 2.4 | 0.0 | 0.0 | 0.0 | 2.7 | 0.0 | 0.0 | 0.7 | 68.3 |
| " | 50 | 1931 | 1 | ..... | 0.0 | 3.4 | 1.5 | 1.0 | 0.0 | 0.0 | 0.6 | 0.3 | 1.2 | 1.0 | 35.4 |
| Harper | 20 | 1929 | 3 | 3.4 | 0.0 | 0.4 | 0.8 | 1.6 | 1.6 | 1.2 | 1.8 | 0.5 | 3.2 | 0.2 | 47.4 |
| Jewell | 21 | 1929 | 3 | 0.0 | 0.0 | 0.3 | 1.8 | 1.9 | 0.0 | 0.1 | 0.1 | 0.4 | 0.3 | 0.0 | 40.9 |
| Kiowa | 29 | 1930 | 2 | 2.6 | 0.4 | 0.6 | 0.2 | 1.2 | 0.2 | 0.6 | 1.2 | 0.0 | 3.3 | 0.0 | 51.1 |
| Lane | 54 | 1931 | 1 | 0.8 | ..... | 0.7 | 0.4 | 0.6 | 0.0 | 0.0 | 1.0 | 1.3 | 6.8 | 0.0 | 65.8 |
| " | 51 | " | 1 | 0.5 | 1.0 | 0.0 | 0.9 | 1.3 | 0.0 | 0.0 | 1.9 | 1.0 | 3.5 | 0.4 | 60.8 |
| " | 52 | " | 1 | 0.4 | 0.0 | 0.0 | 1.3 | ..... | 0.0 | ..... | 0.0 | ..... | 1.4 | 0.0 | 33.7 |
| Lincoln | 30 | 1930 | 2 | 0.0 | 0.4 | 0.3 | 1.8 | 0.3 | 0.0 | 0.0 | 2.1 | 0.2 | 5.0 | 0.0 | 60.5 |
| " | 55 | 1931 | 1 | 0.0 | 0.0 | 0.0 | 2.1 | 3.0 | 0.0 | 0.0 | 1.4 | 0.0 | 3.5 | 0.0 | 68.2 |
| McPherson | 9 | 1928[a] | 1 | 0.0 | 0.0 | 3.1 | 0.0 | 0.6 | 0.0 | 0.0 | 1.8 | 0.0 | 5.9 | 1.2 | 85.5 |
| Meade | 10 | 1928[a] | 1 | 0.0 | 0.0 | 1.8 | 1.5 | 0.2 | 0.0 | 0.0 | 1.5 | 0.0 | 3.4 | 2.3 | 77.8 |
| Mitchell | 57 | 1931 | 1 | 5.3 | 0.0 | 1.5 | 1.1 | 0.3 | 0.0 | 0.4 | 0.7 | 0.0 | 1.1 | 0.0 | 39.5 |

TABLE 2.—*(Continued)*

| Source of smut | Collection No. | Year collected | Number of years tested | Average percentage of smutted heads | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Banner Berkeley | Hussar | Oro | Ridit | Turkey Sel. 1558A | Martin | White Odessa | Kooperatorka | Turkey × Bearded | Yogo | Regal | Kanred |
| *County* | | | | *Physiologic Form 1—(Cont.)* | | | | | | | | | | | |
| Ness | 11 | 1928[a] | 1 | 0.2 | 0.0 | 0.0 | 0.0 | 1.4 | 0.0 | 2.2 | 6.5 | 0.2 | 2.6 | 3.7 | 72.1 |
| Norton | 58 | 1931 | 1 | 1.9 | 0.5 | 1.5 | 1.6 | 1.4 | 0.0 | 0.0 | 1.2 | 0.0 | 1.3 | 0.0 | 46.5 |
| Reno | 31 | 1930 | 2 | 1.0 | 0.6 | 0.0 | 0.9 | 2.4 | 0.0 | 0.0 | 0.4 | 0.3 | 1.4 | 0.0 | 48.4 |
| Rooks | 32 | 1930 | 2 | 0.3 | 1.6 | 0.2 | 0.9 | 0.3 | 0.0 | 0.0 | 2.3 | 0.7 | 2.6 | 0.4 | 53.5 |
| Russell | 64 | 1931 | 1 | ..... | 1.4 | 1.1 | 0.5 | 0.0 | 0.5 | 0.0 | 1.8 | 0.3 | 0.0 | 0.0 | 50.9 |
| Sheridan | 60 | 1931 | 1 | 0.0 | 0.4 | 0.3 | 0.9 | 1.7 | 0.0 | 0.0 | 1.0 | 0.7 | 1.9 | 0.0 | 39.7 |
| Sherman | 62 | 1931 | 1 | 1.1 | 1.1 | 0.0 | 1.4 | 2.5 | 0.9 | 0.0 | 1.5 | 0.0 | 2.8 | 1.5 | 29.9 |
| " | 63 | " | 1 | 2.7 | 0.0 | 0.0 | 1.5 | 2.0 | 0.0 | 0.0 | 0.3 | 0.3 | 0.0 | 0.0 | 23.6 |
| Smith | 24 | 1929 | 2 | 0.6 | 0.0 | 0.0 | 0.6 | 1.5 | 0.0 | 0.0 | 2.4 | 0.3 | 0.9 | 0.0 | 42.5 |
| " | 34 | 1930 | 1 | 2.4 | 0.0 | 0.0 | 3.5 | 0.0 | ..... | 0.0 | 1.5 | 0.6 | 0.8 | 0.0 | 73.2 |
| " | 35 | " | 2 | 0.0 | 0.0 | 0.2 | 0.9 | 1.0 | 0.0 | 0.0 | 1.9 | 1.0 | 0.9 | 0.0 | 50.4 |
| Sedgwick | 33 | 1930 | 2 | 1.7 | 0.2 | 0.4 | 0.0 | 2.1 | 0.2 | 0.0 | 0.4 | 0.0 | 1.6 | 0.0 | 47.3 |
| Sumner | 25 | 1929 | 3 | 0.0 | 0.7 | 0.5 | 1.1 | 1.5 | 0.0 | 0.0 | 1.6 | 0.4 | 0.1 | 0.1 | 34.3 |
| Thomas | 15 | 1928[a] | 1 | 4.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 2.1 | 1.4 | 41.4 |
| | | | | *Physiologic Form 2* | | | | | | | | | | | |
| Brown | 17 | 1929 | 3 | 27.5[b] | 0.0 | 0.0 | 0.0 | 1.4 | 14.6 | 11.5 | 8.7 | 0.8 | 0.2 | 6.0 | 17.4 |
| Johnson | 8 | 1928 | 4 | 33.2 | 0.5 | 3.8 | 0.2 | 1.8 | 12.8 | 8.9 | 17.4 | 0.5 | 0.9 | 4.0 | 35.6 |
| | | | | *Physiologic Form 3* | | | | | | | | | | | |
| Hamilton | 28 | 1930 | 2 | 4.0 | 4.6 | 0.0 | 0.0 | 1.4 | 1.9 | 3.6 | 0.9 | 0.8 | 2.2 | 0.3 | 33.8 |
| Meade | 23 | 1929 | 3 | 2.0 | 7.2 | 0.1 | 3.7 | 3.9 | 5.5 | 5.4 | 0.9 | 0.0 | 2.2 | 0.0 | 54.4 |
| | | | | *Physiologic Form 4* | | | | | | | | | | | |
| Edwards | 49 | 1931 | 1 | ..... | 1.8 | 0.7 | 1.9 | 8.9 | 0.0 | 0.5 | 5.5 | 0.0 | 3.4 | 0.0 | 61.0 |
| Lane | 22 | 1929 | 3 | 0.0 | 0.7 | 0.1 | 0.9 | 3.7 | 0.0 | 0.2 | 1.6 | 0.6 | 1.2 | 0.0 | 38.4 |
| Pawnee | 12 | 1928[a] | 1 | 0.2 | 0.0 | 0.0 | 0.5 | 6.2 | 0.0 | 0.0 | 0.6 | 0.8 | 3.8 | 0.7 | 82.3 |
| Pratt | 14 | 1928[a] | 1 | 0.0 | 0.0 | 0.0 | 0.0 | 14.4 | 0.0 | 0.0 | 1.6 | 0.0 | 0.0 | 0.0 | 53.4 |
| Reno | 13 | 1928[a] | 1 | 0.0 | 0.0 | 0.9 | 0.0 | 6.6 | 0.0 | 0.0 | 0.0 | 0.0 | 3.0 | 0.0 | 60.5 |
| Scott | 36 | 1930 | 2 | 3.2 | 0.7 | 0.0 | 0.0 | 4.2 | 0.0 | 0.0 | 2.0 | 0.2 | 0.5 | 0.8 | 50.4 |

TABLE 2.—*(Continued)*

| Source of smut | Collection No. | Year collected | Number of years tested | Average percentage of smutted heads | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Banner Berkeley | Hussar | Oro | Ridit | Turkey Sel. 1558A | Martin | White Odessa | Kooperatorka | Turkey × Bearded | Yogo | Regal | Kanred |
| *County* | | | | *Physiologic Form 5* | | | | | | | | | | | |
| Barton | 1 | 1928[a] | 1 | 0.0 | 0.0 | 3.1 | 5.7 | 0.2 | 0.0 | 0.2 | 0.4 | 0.0 | 2.8 | 0.4 | 61.9 |
| " | 2 | 1928[a] | 1 | 0.0 | 0.0 | 0.6 | 11.2 | 0.0 | 0.0 | 0.0 | 1.5 | 0.0 | 2.2 | 2.5 | 59.4 |
| Clark | 3 | 1928[a] | 1 | 0.0 | 0.0 | 2.4 | 10.4 | 0.0 | 0.0 | 0.3 | 2.3 | 0.0 | 4.5 | 0.2 | 41.6 |
| Comanche | 4 | 1928[a] | 1 | 0.0 | 0.0 | 0.6 | 5.1 | 0.8 | 0.0 | 0.0 | 5.6 | 0.0 | 1.3 | 2.1 | 60.4 |
| Pratt | 59 | 1931 | 1 | 0.0 | 2.4 | 0.0 | 5.5 | 0.3 | 0.5 | 0.0 | 0.3 | 0.0 | 2.2 | 0.0 | 44.4 |
| Sheridan | 61 | 1931 | 1 | 0.0 | 0.0 | 1.2 | 5.2 | 0.0 | 0.0 | 0.0 | 1.0 | 1.3 | 2.7 | 0.0 | 33.8 |
| | | | | *Physiologic Form 6* | | | | | | | | | | | |
| Lincoln | 56 | 1931 | 1 | 6.3 | 2.6 | 8.7 | 2.1 | 3.6 | 1.0 | 0.4 | 1.0 | 1.2 | 7.4 | ..... | 73.2 |
| | | | | *Physiologic Form 7* | | | | | | | | | | | |
| Greeley | 19 | 1929 | 3 | 10.9 | 0.2 | 0.3 | 0.9 | 1.4 | 1.2 | 2.7 | 1.5 | 0.5 | 1.8 | 0.4 | 39.1 |
| Lane | 53 | 1931 | 1 | 20.3 | 3.6 | 0.3 | 1.4 | 1.8 | 1.4 | 1.8 | 1.4 | 0.0 | 0.7 | 0.3 | 43.1 |

[a] Tested only one year. Through error, these bunt collections were discarded.

[b] Average for 2 years, 1930–1931, as results for Banner Berkeley, 1932, were lost.

the fact that the bunt percentages were higher in 1929 and 1931 than in 1930 and 1932. It also is evident that certain bunt collections may undergo changes in physiologic-form content from year to year, even though carefully handled. For this reason a collection of bunt may attack a differential variety heavily one year and less so the following year, thereby lowering the average percentage of infection over a series of years. This is evident from table 1.

The data in table 1 indicate that the collections fall rather naturally into 7 groups, according to varietal reactions. Some groups contained collections from many sources, while others contained only a few. After the data were averaged and arranged by forms, as in table 2, it became clear that each form had a few outstanding characteristics by which it could be quickly and readily recognized. These characteristics are as follows:

Physiologic form 1 is characterized by low or zero infection on all varieties except Kanred. Forty-seven collections of bunt are included in this physiologic form.

Physiologic form 2 is characterized by high infection on Banner,

Berkeley and moderately high infection on Martin, White Odessa, and Kooperatorka. Two bunt collections are included in this physiologic form.

Physiologic form 3 is characterized by comparatively high infection on Hussar and a moderate amount of infection on Martin and White Odessa. Two bunt collections are included in this form.

Physiologic form 4 is characterized by moderately high infection on Turkey. This form includes 6 bunt collections.

Physiologic form 5 is characterized by moderately high infection on Ridit and includes 6 bunt collections.

Physiologic form 6 is characterized by moderately high infection on Oro, while Hussar, Martin, and White Odessa are resistant. Only one bunt collection is included in this form.

Physiologic form 7 is characterized by high infection on Banner Berkeley and low infection on Martin, in contrast to physiologic form 2, to which Martin is more susceptible. Two bunt collections are included in this form.

On the basis of the grouping in table 2, a dichotomous key has been prepared for the identification of the physiologic forms occurring in the Kansas collections of bunt tested in the seasons of 1929 to 1932, inclusive. Seven forms are separated by differences in pathogenicity on Banner Berkeley, Hussar, Oro, Ridit, Turkey, and Martin. Regal and Kooperatorka have possibilities as differential varieties and could be used in the identification of certain forms. The six varieties used as differentials adequately separate the forms in these investigations. Yogo and Turkey × Bearded show some resistance to all Kansas collections of bunt tested. The latter especially seems to be the most resistant of any of the varieties grown.

KEY FOR THE IDENTIFICATION OF PHYSIOLOGIC FORMS OF BUNT IN KANSAS

| | *Form No.* |
|---|---|
| Banner Berkeley resistant | |
| Hussar highly resistant | |
| Oro highly resistant | |
| Ridit highly resistant | |
| Turkey Sel. highly resistant | 1 |
| Turkey Sel. moderately susceptible | 4 |
| Ridit moderately susceptible | 5 |
| Oro moderately susceptible | 6 |
| Hussar moderately susceptible | 3 |
| Banner Berkeley susceptible | |
| Martin resistant | 7 |
| Martin susceptible | 2 |

### DISTRIBUTION AND PREVALENCE OF PHYSIOLOGIC FORMS OF BUNT IN KANSAS

The distribution of the physiologic forms of bunt in Kansas, based on the collections tested, is shown in figure 1 (lower map). The collections were made in those counties having county agricultural agents and are in areas where wheat production is an important industry. Not all the State, however, is represented, as it was necessary to make the collections where bunt was readily available and through the aid of the county agents and extension specialists.

It will be observed that form 1 is apparently very widely distributed over the hard red winter-wheat belt of the State and is by far the most common form. Form 2 is confined to the soft red winter-wheat sections of northeastern Kansas. Further studies are needed to show just how common or widely distributed it is. Form 3 appears in 2 counties in southwestern Kansas, while forms 4 and 5 are found distributed over the south-central portion of the State. Form 6 was found only in Lincoln county in the central part of the State, and form 7 occurs in 2 of the west-central counties.

### REINOCULATING VARIETIES OF WHEAT WITH THEIR OWN BUNT

In connection with a study of the physiologic forms of bunt, an attempt was made to ascertain how these forms might have originated and how they might have increased. Dillon Weston holds that resistant varieties "break down" in their resistance to bunt (21). This same investigator and Bressman (2) have been able to increase the percentage of bunt in Ridit by inoculating seed of this variety with bunt obtained from smutted Ridit heads.

Reinoculation experiments were begun at the Kansas Agricultural Experiment Station in 1929 in which such resistant varieties as Hussar, White Odessa, Yogo, Turkey × Bearded, Kooperatorka, Martin, and Ridit were used. In cases where traces of bunt occurred on these varieties, as grown in the bunt nursery, the bunted heads were saved and used as inoculum in the reinoculation of the same variety the following year. By this method it should be possible to determine whether this bunt is especially virulent on a variety regarded as resistant. If so, this form of bunt should increase in abundance on that variety in succeeding years. These studies showed that in some instances a strain of smut increased, while in others it seemed to become innocuous and finally disappeared. The results of these reinoculations are given in tables 3 and 4. It will be noted that by reinoculating certain varieties the smut percentages were increased during the course of the studies. Significant increases over 1930 infections are to be noted in 10 cases out of 32 (Table 3). In one case (Turkey

TABLE 3.—*Varieties showing small percentages of bunt infection in 1929, which were reinoculated with their own smut in 1930–1932. Manhattan, Kansas*

| 1930 Culture No. | Varietal source of bunt | County source of bunt 1929 | Percentage of bunt when reinoculated | | |
|---|---|---|---|---|---|
| | | | 1930 | 1931 | 1932(a) |
| 107 | Turkey × Bearded | Barton | 2.5 | 1.8 | ..... |
| 112 | Kooperatorka | " | 1.0 | 0.3 | ..... |
| 116 | Martin | Brown | 2.2 | 24.9 | ..... |
| 117 | White Odessa | " | 2.5 | 23.1 | ..... |
| 122 | Banner Berkeley | " | 23.1 | 57.9 | 27.9 |
| 123 | Regal | " | 2.5 | 12.3 | ..... |
| 129 | White Odessa | Gove | 0.9 | 0.0 | ..... |
| 131 | Turkey × Bearded | " | 2.5 | 0.0 | ..... |
| 132 | Yogo | " | 0.6 | 1.2 | ..... |
| 136 | Kooperatorka | " | 1.0 | 0.0 | ..... |
| 148 | Kooperatorka | Greeley | 0.8 | 0.0 | ..... |
| 149 | Turkey 1558A | Harper | 4.2 | 3.6 | ..... |
| 152 | Martin | " | 1.0 | 8.0 | ..... |
| 155 | Turkey × Bearded | " | 0.6 | 0.8 | ..... |
| 156 | Yogo | " | 1.1 | 0.0 | ..... |
| 166 | Oro | Jewell | 0.4 | 0.0 | ..... |
| 167 | Turkey × Bearded | " | 0.3 | 2.5 | 0.0 |
| 177 | White Odessa | Johnson | 1.0 | 11.6 | ..... |
| 182 | Banner Berkeley | " | 7.5 | 55.2 | ..... |
| 185 | Turkey 1558A | Lane | 4.1 | 0.0 | ..... |
| 193 | Ridit | " | 0.5 | 2.4 | 3.8 |
| 196 | Kooperatorka | " | 1.6 | 1.4 | 0.0 |
| 197 | Turkey 1558A | Meade | 5.1 | 6.0 | ..... |
| 199 | Hussar | " | 7.4 | 47.2 | 16.6 |
| 200 | Martin | " | 1.7 | 23.5 | ..... |
| 201 | White Odessa | " | 1.1 | 37.9 | 15.5 |
| 204 | Yogo | " | 3.4 | 0.9 | ..... |
| 205 | Ridit | " | 0.6 | 14.8 | ..... |
| 220 | Kooperatorka | Smith | 1.8 | 2.3 | ..... |
| 221 | Turkey 1558A | Sumner | 2.1 | 3.4 | ..... |
| 223 | Hussar | " | 2.2 | 2.3 | ..... |
| 232 | Kooperatorka | " | 0.8 | 2.4 | ..... |

(a) The bunt percentages were much lower for even the most susceptible varieties in 1932, so that the percentages in this column may be considered significant.

Sel. culture No. 185, Lane County smut) the percentage was significantly reduced. This is not unexpected. There is the possibility that the smut obtained in the variety came from a plant that was not typical of the variety. Naturally, one would not expect this bunt to increase on the typical variety if the smut were used the following year. It may also happen that the smut present on a resistant variety consists of one or only

TABLE 4.—*Reinoculating a strain of bunt on a normally resistant variety of wheat, thereby increasing the percentage of infection*

| Source of bunt Kans., 1929 | Bunt collection number | Year of Backinoculation | Percentage of bunted heads | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Banner Berkeley | Hussar | Oro | Ridit | Turkey | Martin | White Odessa | Kooperatorka | Turkey × Bearded | Yogo | Regal | Kanred |
| *County* | | | | | | | | | | | | | | |
| Brown | 17 | 1930 | **23.1** | 0.0 | 0.0 | 0.0 | 1.0 | 2.2 | 2.1 | 4.3 | 1.0 | 0.0 | 2.5 | 25.3 |
| Reinoculated | 17a | 1931 | **57.9** | | | | | | | | | | | |
| on Banner Berkeley | 17a | 1932 | **27.9** | 0.4 | 0.3 | 1.4 | 0.5 | 3.0 | 7.2 | 5.0 | 0.0 | 2.2 | 3.3 | 6.0 |
| Meade | 23 | 1930 | 0.0 | **7.4** | 0.0 | 0.6 | 5.1 | 1.7 | 1.1 | 0.0 | 0.0 | 3.4 | 0.0 | 45.1 |
| Reinoculated | 23a | 1931 | | **47.2** | | | | | | | | | | |
| on Hussar | 23a | 1932 | 69.4 | **16.6** | 0.7 | 1.4 | 1.6 | 15.5 | 10.5 | 17.7 | 2.9 | 2.7 | 6.7 | 19.5 |
| Meade | 23 | 1930 | 0.0 | 7.4 | 0.0 | 0.6 | 5.1 | 1.7 | **1.1** | 0.0 | 0.0 | 3.4 | 0.0 | 45.1 |
| Reinoculated | 23b | 1931 | | | | | | | **37.9** | | | | | |
| on White Odessa | 23b | 1932 | 43.8 | 14.6 | 2.8 | 0.0 | 1.9 | 3.1 | **15.5** | 14.1 | 0.0 | 4.5 | 6.4 | 35.4 |

a part of a single bunted head, perhaps on a late, small, poorly developed tiller. The smut dosage has a direct bearing on the percentage of bunt produced, and, if the amount of inoculum has to be spread too thinly over the seed for a row five feet in length, there may be no smut in the crop. This may be the explanation for the low infections of bunt in cultures 129, 131, 136, and 148 in 1930 (Table 3).

The varieties listed in table 3 are, generally speaking, resistant to bunt in Kansas, especially when they are compared with Kanred and Blackhull. The results of the reinoculation studies in 1932 are given for a 3-year period in 6 of the 32 cultures used in these studies in 1930 and 1931. These 6 cases included 3 that had shown rather high smut percentages and 3 that were low. No attempt has been made to see how many new physiologic forms might be demonstrated by this "straining method" or building up of the inoculum on resistant varieties. Banner Berkeley culture No. 122, Hussar culture No. 199, and White Odessa culture No. 201 gave high percentages of bunt. The results seem especially interesting in the case of Hussar, in which the bunt has increased from 7.4 to 47.2 per cent in one year, and in White Odessa culture No. 201, in which the bunt increased from 1.1 to 37.9 per cent. In contrast to these, Ridit culture No. 193 had 0.5 per cent in 1930 and 3.8 per cent in 1932, a very small increase of doubtful significance. A more clear-cut demonstration of what may occur in reinoculation studies is exhibited in table 4. Three collections of bunt

were made in 1929 and used to inoculate differential varieties with results as shown for 1930. In 1931 Banner Berkeley, Hussar, and White Odessa were each reinoculated with their own smut. The smut produced in 1931 on those 3 varieties was again used to inoculate the entire series of differential varieties in 1932.

Banner Berkeley, Hussar, and White Odessa are considered resistant varieties, at least to the majority of bunt collections of Kansas. Obviously, however, collections of bunt do occur that contain forms that attack these varieties. It also is clear that the percentages of infection may increase over a period of years if those forms are allowed to develop on the susceptible host variety. The season of 1932 was not favorable for heavy bunt infection, which explains why the percentages dropped from the high points of 1931 to lower percentages in 1932. Hussar had 7.4 per cent bunt in 1930, which resulted from inoculum applied from bunt collected in Meade County in 1929. This bunt from Hussar was reinoculated on Hussar and gave 47.2 per cent of smut in 1931. In 1932 this smut was applied not only to Hussar, but also to the other standard varieties. By comparing the reactions of the standard varieties to bunt in 1930 and 1932, it will be seen that Banner Berkeley, Hussar, Martin, White Odessa, Kooperatorka, and Regal show increased susceptibility.

Striking differences also may be noted in the reaction of White Odessa to the same collection of bunt. The same procedure of reinoculating White Odessa with its own bunt resulted in marked increases the following year on Banner Berkeley, Hussar, Kooperatorka, and Regal. These results fully justify the conclusion that the occurrence of a small percentage of bunt, such as 7.4 or even 1.1, on certain resistant varieties may be due to the presence of a distinct physiologic form occurring as a slight mixture in the inoculum.

On the other hand, it also is true that the appearance of small quantities of bunt on a resistant variety may not indicate the presence of a virulent physiologic form. Turkey × Bearded had 0.3 per cent infection from culture No. 167 in 1930 and none in 1932. Kooperatorka had 1.6 per cent bunt from culture No. 196 in 1930 and none in 1932.

The results given in tables 3 and 4 leave little or no question that physiologic forms of smut may be built up in this manner. This makes it appear that a resistant variety may become susceptible and physiologic forms may be increased and spread by the introduction and increase of a new variety of wheat in a locality.

### CULTURAL STUDIES OF PHYSIOLOGIC FORMS OF BUNT

It is possible to learn about the characteristics of physiologic forms of smut in several ways; namely, studies of cultural characteristics on arti-

ficial media, pathogenicity, physico-chemical properties, and, to a limited extent, morphologic differences, although the latter are less reliable. Since there are outstanding differences in the physiologic forms of *Tilletia laevis* as to their reaction on certain varieties of wheat, it seemed desirable to study their growth habits on artificial media.

Without attempting to record the details of the culture preparations, isolations of spores, and general technique, it will suffice to say that plain agar, soil-extract agar, and wheat (grain)-extract agar are satisfactory for the germination of chlamydospores of *Tilletia laevis* and for the growth of colonies of the fungus. Potato-sucrose agar (4 per cent) and oatmeal-dextrose (3 per cent) agar were found to be the best media. Five physiologic forms were found to show some differences in color, type of margin, topography, surface growth, and consistency of the colony (Fig. 2). They were selected on the basis of showing cultural growth differences. These forms are strikingly different also in the range of wheat varieties that they attack.

The cultures were made by isolating colonies of germinating chlamydospores. Cultures from single primary sporidia were not obtained, since it was difficult to make them grow after they were isolated. This is not the case with the sorghum kernel smut, *Sphacelotheca sorghi*, and some of the other smuts. The cultures described herein, therefore, are not so reliable for cultural study as those obtained from single sporidia, because of the phenomenon of heterothallism. However, as far as could be noted, the cultures obtained in this manner were quite consistent in their characteristics, even after several transfers were made. It was felt, however, that not a great deal of emphasis should be laid on the results obtained in the study of the bunt organism on artificial media, at least in so far as separating physiologic forms is concerned.

The studies soon revealed that *Tilletia laevis* develops very slowly, and the colonies, therefore, were grown in Erlenmeyer flasks to avoid rapid drying. The cultures were grown at temperatures ranging from 17° to 20° C. for a period of 2 months. The 5 physiologic forms, as grown on potato-sucrose agar, are described in table 5.

It was not possible to secure satisfactory cultures of all of the 7 forms; therefore, the record is given for only the 5 forms that were obtained. The cultures were separated by their growth and color differences. It was apparent, that the several forms cultured did not always produce "typical growth" characteristics each time they were grown from chlamydospores. This is not surprising in view of the recent studies by Flor (7), who obtained great variation, even among monosporidial cultures within physiologic forms. Also, the importance that should be attached to the cultural studies of bunt has been questioned by this same writer (7).

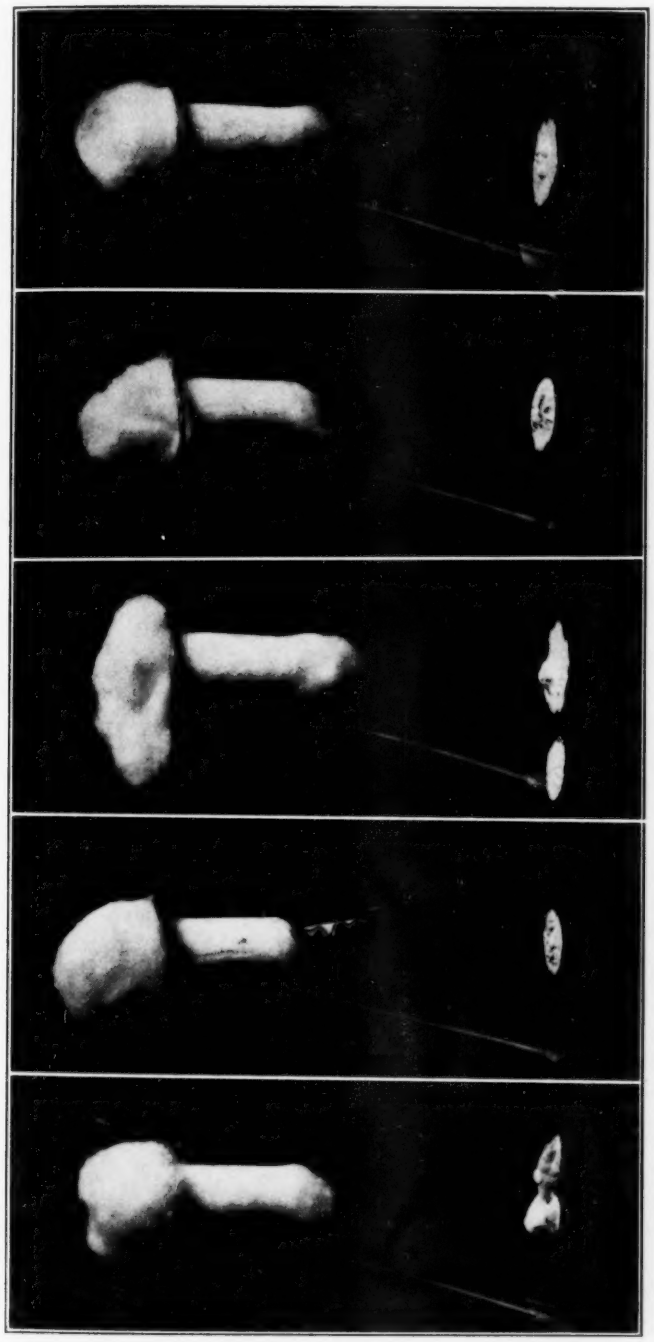

FIG. 2. Physiologic forms 1 to 5, of *Tilletia laevis*. Their cultural characteristics are described in table 5.

TABLE 5.—*Cultural characteristics of Tilletia laevis two months after planting. May 13, 1931*

| Physiologic form | County source of smut | Year collected | Diam. of culture in mm. | Color | Consistency | Colony characters | Color of medium |
|---|---|---|---|---|---|---|---|
| 1 | Greeley | 1929 | 15 × 10 | Greyish. | Plastery to cottony. | Raised 10 mm.; margin very irregular; no exudate droplets; sporidial discharge; circular furrows around cottony mound. | Unchanged. |
| 2 | Johnson | 1928 | 23 × 20 | Bluish-white, few white cottony knobs. | Velvety. | Flat, except for few scattered knobs; margin irregular and definite; no sporidial discharge; no exudate droplets; surface furrowed. | Slightly brown throughout. |
| 3 | Meade | 1929 | 25 | White. | Velvety with cottony margins. | Raised 6 mm.; margin irregular and definite; irregular mounds at center; sporidial discharge; few exudate droplets light brown; surface irregular and furrowed. | Slightly brown around colony. |
| 4 | Lane | 1929 | 21 | Greyish. | Cottony. | Flat, except for knobs bearing exudate droplets at center; colony nearly circular; margin irregular and definite; surface slightly furrowed. | Deep brown throughout. |
| 5 | Barton | 1928 | 26 | Pure white. | Velvety with cottony margins. | Raised 4 mm.; few plaster-like, white knobs at the center; margin irregular and definite; no sporidial discharge; few exudate droplets; surface of colony slightly furrowed. | Unchanged. |

### A PROGRAM OF BREEDING FOR BUNT RESISTANCE IN WINTER WHEAT

The difficulties, due to the existence of physiologic forms, in breeding for bunt resistance, have been mentioned herein and elsewhere (2). The task should not be considered as hopeless, however. The fact that there are some resistant varieties or selections in each of the groups of wheat, *i.e.*, in the 42-, the 28-, and the 14-chromosome groups, indicates that resistance is not specifically correlated with any gross morphologic type. This allows considerable liberty in the selection of resistant parental stock and greatly increases the chances of success. It should be possible, therefore, to secure a commercially adapted winter wheat for Kansas that is resistant to bunt. This should be possible by either selection or hybridization, but the possibilities of both methods should be fully explored. The promising resistant selections of Turkey wheat recently made in Nebraska (11) well illustrate the possibilities in that direction. A cooperative program for breeding for bunt resistance is under way at the Kansas Agricultural Experiment Station and some promising hybrid selections have been made, especially from the cross Oro × Tenmarq.

The greater the range of the bunt collection to which the wheat selections or hybrids are subjected, the more dependable the final results will be. Even if tested to a number of forms of bunt, however, the resistance of a variety will probably be maintained only for a relatively short period of years. The greater the area over which the variety is to be grown, the greater the likelihood of encountering a physiologic form or forms that will attack the new variety. There also is the possibility that forms may be introduced from other states or counties. This problem must be faced by the pathologist and plant breeder, who should be cautious about making positive statements regarding the resistance of new varieties. There will then be fewer disappointments on the part of the research worker, and less criticism from administrators and from farmer taxpayers who support research in plant breeding and plant pathology. It is not likely that each new physiologic form that may be encountered will seriously change the picture. However, existing physiologic forms will gradually find their way into the region where a newly produced variety is grown and possibly alter the situation. How fast this may take place is a question needing further study. It should be recognized, however, that each new physiologic form discovered, or produced by hybridization or mutation, does not necessarily complicate the problem of breeding for resistance, since it is known that the resistance to certain groups of physiologic forms is inherited in a comparatively simple manner.

In a program of breeding for bunt resistance, the testing of the selections, varieties, or hybrids should consist of inoculating the seed with as many physiologic forms or collections of bunt as can be secured in the State

or region in which the varieties may be grown. This is not a new suggestion, but the author wishes to emphasize its importance. Later, the variety, selection, or hybrid may be planted in a series of uniform bunt nurseries where it will be inoculated with many forms of bunt and grown under varying environmental conditions. This method of testing represents more nearly what the variety is likely to encounter under conditions of wide distribution. New collections of bunt should be added to the forms already in use as the variety testing progresses. With this procedure, the actual reaction to bunt is more likely to be learned.

It is not a simple matter to determine the resistant parents to be used in crosses, because the literature shows that numerous varieties that at one time were regarded as resistant to bunt are no longer resistant to all forms of bunt (2, 10). This greatly limits the choice of varieties useful in hybridization. The truth of this is realized when one considers that varieties such as White Odessa, Albit, Regal, Martin, Hussar, Banner Berkeley, Oro, and Ridit, which have been called resistant or immune, are now known to be susceptible to one or more physiologic forms of the bunt organism as shown in these studies and by Bressman (2).

## SUMMARY

Increasingly severe outbreaks of wheat bunt in Kansas in recent years indicate the presence of virulent strains of the bunt organism.

Tests were conducted in the bunt nursery of the Department of Botany at Manhattan, Kansas, over a 4-year period to determine the reaction of 12 selected varieties of winter wheat to many collections of bunt from various counties in the important wheat-growing sections of the State.

All collections of Kansas bunt were found on microscopic examination to belong to the species, *Tilletia laevis*.

Distinct differences in virulence among the bunt collections were manifested on the varieties Turkey (C.I. 1558A), Martin (C.I. 4463), Hussar (C.I. 4843), White Odessa (C.I. 4655), Oro (C.I. 8220), Ridit (C.I. 6703), and Banner Berkeley (C. I. 7362).

The collections of bunt have been grouped according to their types of virulence on these varieties into 7 physiologic forms. A dichotomous key for use in the separation of forms is presented in the text.

The varieties Turkey × Bearded (C.I. 8243) and Yogo (C.I. 8033) show resistance to all collections of Kansas bunt so far tested. Martin (C.I. 4463), Ridit (C.I. 6703), Hussar (C.I. 4843), White Odessa (C.I. 4655), Banner Berkeley (C.I. 7362), Regal (C.I. 7364), Oro (C.I. 8220), and Kooperatorka (C.I. 8861) show considerable resistance to most collections of bunt in Kansas, but the majority are known to be somewhat susceptible to certain forms in other parts of the United States.

A map is included showing the distribution of the 7 physiologic forms of *Tilletia laevis* in Kansas. Form 1 is the most widely distributed.

Reinoculating bunt on resistant varieties of wheat that showed small percentages of bunt the previous season resulted in an increased infection in 10 cases out of 32. Very decided increases in infection on certain resistant varieties suggest that new forms may have developed by segregation following hybridization.

Cultural studies have shown that plain agar, soil-extract agar, and wheat (grain)-extract agar are satisfactory for the germination of chlamydospores of *Tilletia laevis* and for the initiation of colony growth.

The best media for showing cultural differences among physiologic forms of bunt were found to be potato-sucrose (4 per cent) and oatmeal-dextrose (3 per cent) agar. Consistent and distinct differences in cultural characteristics were shown to exist among representative bunt collections of five physiologic forms.

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# TREATMENT OF SWEET-POTATO PLANTS FOR THE CONTROL OF BLACK ROT

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Sweet potatoes constitute one of the most important commercial crops in Mississippi. In 1932 the total production for the State was in excess of 7,900,000 bushels. Only Georgia, Alabama, and North Carolina exceeded this amount for that year. Now, with so large an acreage of land diverted from cotton to the growing of other crops, it is probable that a still greater production may eventuate in the immediate future.

A long and continued cropping of sweet potatoes without crop rotation and, at least in the first years of the industry, without much attention to sanitary measures to prevent disease dissemination, has resulted in a large amount of "disease-sick soil," that is, soil containing great numbers of sweet-potato pathogens.

Sweet-potato black rot, caused by the fungus, *Ceratostomella fimbriata* (E. & H.) Elliott, is one of the worst diseases with which the grower has to contend. It is both a seed-bed and field disease, and as a storage trouble, it is especially destructive. Although control measures for the disease on the potato and in storage have been worked out rather satisfactorily, the grower of sweet-potato plants for sale purposes still has much trouble with it. In spite of all possible precautions he is likely to have a small amount of it develop in his plant beds. A single infected plant in a bundle of otherwise perfectly clean and healthy plants may, during shipment to the purchaser, serve to contaminate and infect the entire bundle in which the diseased plant occurs, or even the entire shipment. With the system of terminal inspection of parcel post and other shipments now in vogue in many States, such spread of infection in transit results in the condemnation and destruction or the rejection and return to the grower of many large lots of plants that, when packed and shipped by the grower, probably were apparently clean or at that time contained only a trace of infection. If such infection, limited at shipment to a single plant or so, could be held at that point without further spread during transit, the loss of plants by condemnation of shipments and the damage suffered by the purchaser when such lots escape such condemnation, due to escape from or lack of requirement of such terminal infection, would be reduced many hundredfold.

The grower who receives infected plants suffers threefold injury therefrom: (1), the death of infected plants that die without becoming established; (2), the infected plants if they live, will produce black-rotted potatoes; and (3), whether they live or not, will give rise to wholesale soil

infestation that will render the soil unfit for future crops of sweet potatoes. This third consequence is by far the most serious, since the pathogen will persist in the infested soil for an indeterminate number of years even in the absence of sweet-potato crops.

For years growers have been making enquiries indicating a desire for some form of plant treatment that might eliminate or at least check the dissemination of the black-rot organism while the plants are in transit from grower to purchaser. In view of these desires of the growers and in the light of reports of work done in recent years at the North Carolina Agricultural Experiment Station by Poole and Woodside,[1] who have shown that by dipping the stems and roots of sweet-potato plants in concentrated chemicals, infection by the sweet-potato wilt or stem-rot organism, *Fusarium batatatis* Wr., might be prevented without killing the plant, it was decided to make some preliminary tests. Although a diverse assortment of materials and several strengths of Bordeaux mixture were tested by the North Carolina workers, their final conclusion was that a 20–20–50 Bordeaux mixture and a 25 per cent monohydrated copper sulphate-lime dust gave best results, with least injury to the plants treated. In their work, however, the plants were treated immediately prior to being transplanted to the field.

## EXPERIMENTS IN 1932

In the spring of 1932 a few tests were designed to determine the degree to which sweet-potato plants would withstand the treatments mentioned in the foregoing when applied by the grower prior to their shipment to the purchaser. Plants to be used were purchased from a Mississippi grower and were of A-Grade quality, according to the certification system of the State Plant Board of Mississippi. The A-Grade certificate indicated that no black rot or stem rot whatsoever had ever been found on the premises of the grower, either in the field, storage, or plant-bed inspections as made by the regular State Plant Board inspectors.

One hundred plants of the Nancy Hall variety were dipped, stem and roots, in a 20–20–50 Bordeaux mixture solution and then allowed to drain. They were then tied up in bundles of 25 plants each. These 4 bundles were then wrapped together in a piece of newspaper, the tops alone remaining exposed. A similar number of plants were dipped into a copper-lime dust containing 25 per cent monohydrated copper sulphate. It was found unnecessary to moisten the plants, since the dust adhered to the stems and roots in surprisingly large amounts. These, likewise, were tied up in bundles of 25 plants each and wrapped in newspaper, as were the others. An additional 100 plants were divided into bundles and wrapped in a similar man-

[1] Poole, R. F., and J. W. Woodside, A chemical control for sweet potato wilt or stem rot. North Carolina Tech. Bul. 35. 1929.

ner, but with no treatment, to serve as checks. These 300 plants, representing 100 for each of the 2 treatments and 100 for the check, were then packed in a perforated box, such as is customarily used for shipping sweet-potato plants, and the box was set aside on a bench in the laboratory at ordinary room temperature for 5 days. A similar lot of 300 plants were treated and packed in an identical manner 2 days later and allowed to remain in the laboratory for 3 days. These periods of 3 and 5 days were supposed to represent periods of time during which the plants would be in transit from the grower to the purchaser. The 5-day period was chosen as a probable maximum during which plants would ordinarily remain in transit and storage between the time of leaving the grower and the time of being planted out in the field by the purchaser, such as might occur when shipped or received on a week end. The 3-day period was selected as a probable average time for such transit and storage between being pulled by the grower and transplanted to the field by the purchaser.

On June 20, 1932, both lots were planted in the field. The soil of the plot was a rather stiff, dark clay loam, with a small percentage of sand. It was realized that this was not the best type of soil for sweet potatoes, but it was the only land then available. It was rather dry, so that it was necessary to water the plants as they were transplanted. When unpacked the plants that had been held 5 days, packed for shipment, were somewhat yellowish, and the check plants, those not dipped in either the copper-lime dust or the Bordeaux mixture, were somewhat flaccid. The plants whose roots and stems had been dipped in either of these materials seemed considerably more turgid and in better condition generally than did the check plants, stored equally long, but without preliminary treatment. All the plants packed and stored for the 3-day period were green and turgid and in excellent condition for transplanting.

TABLE 1.—*Loss of stand in 1932 as a result of treatment prior to packing for shipment*

Stand on August 2, 45 days after planting

| Treatment | After 3 days in storage | After 5 days in storage | Planted immediately no storage |
|---|---|---|---|
| | *Per cent* | *Per cent* | *Per cent* |
| 20–20–50 Bordeaux mixture | 100 | 98 | 100 |
| 25% Copper-lime dust | 98 | 100 | 100 |
| Check, non-treated | 98 | 97 | 100 |

Although no rain fell until July 2, 13 days after planting, no additional water, except that given at planting time, was ever applied to these plants. Some of the plants in each treatment, including the checks, appeared to be suffering severely. The larger percentage, however, seemed to stand up surprisingly well under the treatment to which they had been subjected. After the rain of July 2 most of the plants that had previously seemed to be dying began to revive and to put out new growth.

It will be noted from the above table that the loss of stand was negligible, even under the extremely severe treatment to which the plants had been subjected. The highest losses, in fact, were in the nontreated plants, excepting those held for 5 days in storage before planting. The next highest losses fell equally on the lots treated with Bordeaux mixture and stored for 5 days, on those treated with copper-lime dust and stored for 3 days, and on the nontreated lot stored for 3 days.

No differences in amount of vine growth throughout the growing season were apparent between the different treatments. Vine growth was exceptionally heavy and remained green and vigorous until late fall. At digging time, however, the yield proved very scanty, irrespective of the treatment accorded the plants. In consequence, no harvest records were taken. The potatoes had, in the parlance of the grower, all "gone to vine." This could not be attributed to any treatment at planting time, since it was common to all, even to those that had received no treatment whatsoever. It is more probable that the abundant vine growth and lack of potatoes were due to the unsuitable nature of the soil on which they were planted and probably in some measure to weather conditions prevalent during the growing season, since the fall season was extremely wet.

The results of this test were interpreted as being extremely favorable, indicating that sweet-potato plants possessed a marked degree of resistance to injury from treatment with highly concentrated copper compounds.

Another test was conducted simultaneously with the one above reported with the object of determining the efficacy of the same copper compounds in the prevention of infection by the black-rot organism. A culture of the black-rot organism, *Ceratostomella fimbriata,* was obtained from a specimen of sweet potato of the variety Porto Rico collected at Kosciusko, Attala County, Mississippi, in August, 1931, and maintained thereafter on a potato-dextrose-agar medium. New cultures were prepared on a cooked sweet-potato medium 4 days before use in inoculation tests. It was found that on the sweet-potato medium there was an abundance of microspores produced for use in making spore suspensions in a very short period of time.

On June 20, 1932, a lot of 100 plants of the Porto Rico variety was inoculated by dipping the roots and stems in a heavy suspension of spores of *Ceratostomella fimbriata* in distilled water. Another 100 plants were used

as checks, being merely dipped into clear distilled water before being planted. Still another 100 plants were first dipped into the spore suspension and, after being allowed to drain off thoroughly, were subsequently dipped as to stems and roots in a 20–20–50 Bordeaux solution before planting. A fourth lot was dipped in the spore suspension and, after drying, immersed into a 25 per cent copper-lime dust.

Thirteen days later, July 12, 100 per cent of the plants inoculated with black rot, but not treated with either of the copper compounds, showed black-rot infection. None of the check plants, dipped in distilled water, nor of the plants inoculated and subsequently treated with either the Bordeaux mixture of the copper-lime dust, ever exhibited any symptom of infection at any time. This test indicated that both treatments were very effective in preventing infection of the plants by surface-borne spores of the black-rot organism. These results, taken in conjunction with those obtained in the previously recorded test, presented such favorable promise of an effective method of prevention of the spread of black rot during transit of plants from grower to purchaser that it was decided to repeat the tests in the spring of 1933 to see whether or not they could be confirmed.

## EXPERIMENTS IN 1933

The same strain of the black-rot organism as that employed in 1932 was used in 1933. New cultures were prepared on a cooked sweet-potato medium a week in advance of inoculation tests, and an abundance of spores were available at that time for making spore suspensions. The soil selected for the test, containing a considerably higher proportion of sand, was much better adapted to the growth of sweet potatoes than was that of 1932. The plants were grown in beds in the greenhouse from A-Grade seed of the Nancy Hall variety. A-Grade seed is apparently clean seed secured from a grower on whose premises no black rot or stem rot has been found, either in plant-bed, field or storage-house inspections, by the authorized inspectors of the Mississippi State Plant Board. The preparation of the soil and cultivation of the crop were similar to the practices commonly in use in commercial sweet-potato culture.

On May 10, 3 lots of 100 plants each were dipped in a heavy suspension of spores of the black-rot organism. After thorough draining, they were wrapped in newspaper, the tops only being left exposed, packed in a shipping box, and stored for 5 days in the laboratory. Three additional lots were dipped in sterile distilled water and wrapped and stored in a similar manner. Six other lots were dipped into the spore suspension. After having drained thoroughly, 3 of them were immersed in a 20–20–50 Bordeaux solution. After having dried, the other 3 were thoroughly dusted with a 25 per cent copper-lime dust. The plants given the Bordeaux treatment had their roots and stems completely immersed in the solution, only the tops re-

maining free from the chemical. Those given the dust treatment were covered tops and all by immersing them entirely in the dust. These lots were then wrapped and stored for five days. On May 12, 3 lots of 100 plants each were subjected to each of the respective treatments described above, the only difference being that the plants treated on this date were placed in storage for 3 days only.

On May 15, all of the plants that had been in storage for the 5- and 3-day periods were removed from storage and transplanted to the field. On many of the plants dipped in the spore suspension and stored for 5 days, visible evidence of infection was already in evidence. Such visible symptoms were not observable on those similarly treated but stored for 3 days only. A small percentage of the plants dipped in the spore suspension and stored for 5 days showed the typical black lesions of black rot, and under the hand lens the seta-like necks of perithecia of the causal organism were visible. None of those dipped in the spore suspension and subsequently treated with either the Bordeaux mixture or the copper-lime dust exhibited any sign of infection.

On May 15, 3 additional lots of 100 plants were subjected to one of each of the treatments described above, with the exception that the storage period was omitted. That is, 3 lots were dipped into the spore suspension, 3 were dipped into sterile distilled water, 3 into the spore suspension and then into the Bordeaux mixture, and 3 into the spore suspension and subsequently into the copper-lime dust. All were then planted simultaneosuly with those lots that had been stored 3 and 5 days, respectively.

The soil was in a finely pulverized condition and so dry at planting time that it was considered necessary to apply a small amount of water about each plant when transplanted to the field. A light rain fell some 10 days later.

As will be seen from table 2, a good stand was secured in all cases except

TABLE 2.—*Infection and stand count as a result of plant treatment, 1933*

| Treatment | Stand, July 1, 45 days after planting | | | Infection, July 1, 45 days after planting | | |
|---|---|---|---|---|---|---|
| | Storage period | | | Storage period | | |
| | none | 3 days | 5 days | none | 3 days | 5 days |
| | *Per cent* | *Per cent* | *Per cent* | *Per cent* | *Per cent* | *Per cent* |
| Check, distilled water | 100 | 100 | 98 | 0 | 0 | 0 |
| Spore suspension | 16 | 17 | 2 | 89 | 100 | 100 |
| Spore suspension and 20–20–50 Bordeaux mixture | 100 | 99 | 98 | 0 | 1 | 0 |
| Spore suspension and 25% copper-lime dust | 99 | 100 | 97 | 1 | 0 | 1.5 |

where the plants were inoculated with the black-rot organism by dipping them in the spore suspension with no subsequent disinfection. In this case the stand was almost destroyed before the end of the 45-day period. At that time the few remaining plants in the plot were destroyed in order to limit as much as possible the spread of the black-rot organism to the disease-free plots. The table shows also that both the 20–20–50 Bordeaux mixture and the 25 per cent copper-lime dust gave almost complete control of black rot, even when the plants were thoroughly inoculated by dipping them in a heavy spore suspension.

Yield records at the end of the season, as shown in table 3, indicate that treatment of plants with either 20–20–50 Bordeaux mixture or the 25 per cent copper-lime dust did not injure the plants beyond their ability to recover under the conditions of the test. In fact, the average yield of the rows treated with these materials and planted immediately was higher in each case, though not beyond the limits of experimental error, than that of the plants merely dipped in distilled water before planting. The yield from the plants treated with 25 per cent copper-lime dust and stored for 3 days was essentially the same as that from plants dipped in water and stored for the same length of time, while those treated with the 20–20–50 Bordeaux mixture and stored for the same period yielded only slightly less. The yield in each case where the plants were stored for 5 days was lower than that from the corresponding treatment, when the plants were planted immediately without undergoing a period of time in shipment or storage. The reduction, however, was nearly as large in the case of the plants dipped in water only prior to storage as in that of plants treated with either Bordeaux mixture or copper-lime dust and stored. It is somewhat of a surprise to note that the yield from plants stored for 5 days was slightly higher than when similarly treated plants were stored for 3 days only. These differences are all very small, however, and are all within the limits of experimental error, hence have no significance.

## DISCUSSION

These results secured from 2 consecutive years of experimentation under field conditions seem to indicate that treatment of sweet-potato plants by the grower, at time of pulling, with either a 20–20–50 Bordeaux mixture or a 25 per cent copper-lime dust may be practiced without material detriment to the plants. The time period of 5 days is ordinarily as long as would occur between treatment by the grower and transplanting by the purchaser. The fact, however, that the yield from plants stored for 5 days was as large as or even larger than from plants stored for 3 days might indicate that the time necessary to shipment and storage might possibly be safely extended even beyond the 5-day period employed in these tests.

TABLE 3.—*Sweet-potato-plant treatment, yield in pounds per 100 plant row, 1933*

| Treatment | No storage | | | | Stored 3 days | | | | Stored 5 days | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ser. 1 | Ser. 2 | Ser. 3 | Ave. | Ser. 1 | Ser. 2 | Ser. 3 | Ave. | Ser. 1 | Ser. 2 | Ser. 3 | Ave. | Ave. yield |
| Check, distilled water | 138.5 | 142.0 | 128.0 | 136.2 | 118.0 | 156.0 | 126.0 | 133.3 | 155.5 | 123.0 | 118.0 | 132.2 | 133.9 |
| Spore suspension and 20–20–50 Bordeaux mixture | 114.5 | 154.0 | 148.0 | 138.8 | 151.0 | 124.0 | 116.0 | 130.3 | 125.0 | 130.5 | 138.5 | 131.0 | 133.4 |
| Spore suspension and 25 per cent copper-lime dust | 127.5 | 168.0 | 126.0 | 140.5 | 137.0 | 118.0 | 146.0 | 133.7 | 104.5 | 137.5 | 151.0 | 131.0- | 135.1 |

The weather conditions in both years of these tests were more unfavorable to the growth of sweet-potato plants at transplanting time than are encountered in the average season, being unusually dry in both cases. Both stand percentages and yield records show that the plants treated with the chemicals are able to withstand these unfavorable conditions almost as well as nontreated plants that have been stored equally long.

These tests also show that either of the above treatments is very efficient in the prevention of infection of the plants by surface-borne spores of the black-rot organism, even under the extremely favorable conditions for infection induced by dipping the plants in a heavy spore suspension of black-rot spores and then subjecting them to long confinement in the close atmosphere of the shipping container. It would seem, therefore, that this is a method whereby spread of black rot on sweet-potato plants may be prevented during the time of shipment from the grower to the purchaser, the interval during which most of such spread normally occurs. It would seem that this method is an adequate answer to the insistent demand on the part of the grower that has been heard so constantly and repeatedly since the advent of sweet-potato-plant inspection, a demand calling for some method whereby this spread of infection may be avoided.

It will be necessary, of course, to educate the purchaser to the acceptance of plants coated with a heavy covering of liquid or dust, which, especially in the case of the Bordeaux mixture, renders them somewhat unsightly. If, however, he can be adequately convinced that such plants will live as well on being transplanted in the field, will produce as well as plants not so treated, and will also yield him a disease-free crop and avoid the introduction into his soil of the persistent and destructive black-rot organism, he will soon become an enthusiastic advocate of such treatments, regardless of the appearance of the plants. If the method works out as well in actual commercial practice as it has in these 2 years of field experimentation, it should prove of great value to the plant producer by eliminating the loss of many shipments that are normally returned to him or destroyed by the plant-inspection agencies. It should result in increased good-will toward him on the part of his customers, since it would eliminate many complaints from disgruntled purchasers that had paid for clean plants and had instead harvested a heavily infected crop of potatoes and, worse still, had destroyed for several years the value of their fields for subsequent sweet-potato production.

The effect of such treatments, when applied to clean plants planted on infested soil, has not yet been determined. Poole has found that such treatments retard, or at least delay very materially, the infection of sweet-potato plants by the stem-rot or wilt organism, *Fusarium batatatis* Wr. By analogy, therefore, it is quite probable that plants so treated would be protected,

at least for a time, and that infection would be delayed in infested soils so that a smaller amount of black-rotted potatoes would result in the harvested crop.

SUMMARY

Tests were made over a period of 2 consecutive years to determine the degree of resistance of sweet-potato plants to treatment with a 20–20–50 Bordeaux mixture solution and with a 25 per cent copper-lime dust. Entire plants immersed in the copper-lime dust and plants with their stems and roots dipped in the concentrated Bordeaux mixture were but slightly affected, either as to stand or yield, when planted immediately after treatment.

Plants inoculated by dipping their stems and roots into a heavy suspension of spores of the sweet-potato black-rot organism, *Ceratostomella fimbriata,* showed a very high percentage of infection when planted immediately. One hundred per cent infection resulted when plants so inoculated were wrapped in bundles and storted for 3- and 5-day periods in a container under conditions such as they would normally undergo during transit from grower to purchaser.

When plants were inoculated by dipping their stems and roots in a black-rot spore suspension and were subsequently treated with either the Bordeaux mixture or the copper-lime dust, infection was almost entirely eliminated, even when the plants were stored in the close atmosphere of the shipping container for periods of 3 and 5 days.

Yields from sweet-potato plants treated with the above materials and stored for 3 and 5 days in a shipping container were but slightly reduced over those plants similarly treated but with the storage period omitted. The yields from plants treated with the chemicals and not stored before planting were slightly higher than from plants merely dipped in water and planted. Plants treated with the disinfectant materials and stored for 3 and 5 days gave no significant difference in yield over plants dipped in water and stored for the same length of time.

These results have made it apparent that sweet-potato-plant growers can treat their plants by dipping them in a 25 per cent copper-lime dust or by immersing their stems and roots in a 20–20–50 Bordeaux solution prior to shipment to the purchaser without materially injuring them, provided the time required for shipment and storage be no longer than 5 days. These tests have not shown the limit of time that plants so treated will endure without injury in storage and transit. Treatment with either of the materials tested will practically eliminate the dissemination and spread of black rot on sweet-potato plants during transit from the grower to the purchaser.

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# SOME PRELIMINARY FEEDING EXPERIMENTS WITH SCABBY BARLEY[1]

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In 1928 barley in the Midwestern States suffered from a severe epidemic of scab caused by *Gibberella saubinetii* (Mont.) Sacc. The same year some of the barley raised in this region was exported to Belgium, Holland, and Germany where it was used for feeding hogs. Grain-exporting firms received complaints, especially from Germany, that the barley was causing disease symptoms in hogs and that such diseased hogs refused to continue eating rations containing this barley.

Scott (9) reported the presence of wheat scab in Iowa as early as 1866, indicating its presence in the State for at least 65 years, and records[3] for the period since 1908 show that a slight amount of scab has been present on barley, except in the years 1919, 1928, and 1932, in which there was a loss in crop yield of from 4 to 5 per cent. It was not until 1928, when there was a loss of 4 per cent, that difficulty was encountered in feeding the grain.

After harvesting the 1928 crop the Iowa Agricultural Experiment Station received numerous complaints regarding the ill effects of feeding scabby barley to hogs in Iowa. Some complaints also were received that chickens would not eat barley and that where chickens ranged in barley fields, either before or after harvest, they became ill.

Grain infected with microorganisms has been known to induce injurious effects to man and animals in Europe and to a lesser degree in this country; but, in the United States, the seriousness of the problem in 1928 exceeded all previous records. This condition of our barley grain led to an investigation, commencing in the fall of 1928, of the effect of scabby barley as a feed for hogs, chickens, and guinea pigs. At the 1929 meeting of the American Phytopathological Society the following results were reported in an abstract by Mundkur and Cochran (5). "Hogs fed on an exclusive diet of this barley developed nausea for food and starved rather than eat it, while checks fed on clean barley were not sick and showed a slight increase in weight. After the fifth day the test animals fed on scabby barley began to vomit, but did not develop diarrhea.

"Mature chickens fed on the scabby barley showed no disease and lost no weight even when they were fed on artificially infected barley; whereas

[1] Journal paper No. J137 of the Iowa Agricultural Experiment Station, Ames, Iowa, Project No. 5. (Old series).

[2] The writer wishes to express his gratitude to Dr. I. E. Melhus for suggestions and assistance in the experimental work and in preparation of the manuscript.

[3] Plant Disease Survey.

chickens two weeks old showed a loss of weight and developed a rough plumage. The birds were not on an exclusive barley diet and it was observed that they rejected a lot of feed, picking out, as far as possible, the mash.

"Guinea pigs rejected an exclusive barley diet. Fed on a half-and-half mixture of scabby barley and mash, they lost in weight, but did not develop any disease symptoms such as was exhibited in the hogs."

At the meeting referred to above, Roche, Bohstedt, and Dickson (8) reported as follows: "The farm utilization of scab-infected barley has been found economical by feeding it to cattle, sheep and poultry. The ruminants and poultry make good gains on heavily scabbed grain with no apparent ill effect. Pigs, horses, and dogs, as well as man, are very sensitive to the accumulated products in the infected grains and will not tolerate low percentages of badly scabbed kernels. No method has been found to date whereby badly scabbed grain can be fed economically to pigs."

Miessner and Schoop (4) noted that when macerated fungus (*Gibberella saubinetii*) material, as well as the liquid in which the fungus had grown, was injected subcutaneously into hogs, it caused violent vomiting and acute paralysis. Mice and guinea pigs did not develop these symptoms, a fact that agrees with the results of feeding experiments with guinea pigs reported in this paper.

In 1930 Roche and Bohstedt (7) found that lambs and ewes showed no apparent difference, whether the grain upon which they were fed was diseased or normal. Scabbed oats carrying 70 per cent infection, if they exceeded 60 per cent of the grain ration, were found to be unpalatable to horses.

In 1930 Mains *et al.* (3) reported that scabby barley had been fed to chickens without causing decrease in weight, but that hogs lost if the amount of scabby barley exceeded 10 per cent. Barley containing 58 per cent scab seemed to have no injurious effect on cattle.

## EXPERIMENTAL STUDIES

*The Causal Organism.*—The diseased grain does not have the attractive pale straw color of healthy bulk barley, but is slightly dark brown in appearance. The tip of the caryopsis is bluish black or very deep brown, which gives the slightly dark brown color to the grain in mass. The grains are shrivelled, and the bluish black tips, when examined with a hand lens, show small black granular bodies occurring in groups on the surface, the perithecia of a pyrenomycetous fungus. The perithecia are ovoid or subconical; each contains over one hundred asci, and each ascus contains eight spores. The fungus was identified as *Gibberella saubinetii* (Mont.) Sacc.

Hyaline hyphae were demonstrated in diseased grains whether they showed perithecia or only brownish tips.

*Tests with Hogs.*[3]—Six hogs were available for experimentation. The initial weight of the hogs was recorded and they were starved for 24 hours. After this compulsory fast they were given rations as follows: Four hogs were given naturally-infected barley showing about 40 per cent of the grains discolored by the scab fungus; 2 hogs serving as checks were given clean, healthy barley.

The infected barley was made into meal and mixed with warm water at the time of feeding. The hogs were fed twice daily. On the first day the feeds were consumed without any apparent hesitation. On the second day the early morning feed was consumed but thereafter it was refused. Substitution of milk for water induced the hogs to consume a part of the feed but they took it reluctantly. After the sixth day they absolutely refused the feed. The hogs fed on clean barley made no objection to their rations. The hogs on scabby barley would not drink even the milk that separated to the top, after the mixture had stood for a while. Vomiting commenced after the sixth day and continued 10 days, when the experiment was terminated, as it was considered inadvisable to starve the animals further. The weights of the animals before and after the experiment are given in table 1.

The losses in weight recorded in table 1 probably should be considered largely due to the starvation of the hogs. The barley induced vomiting but did not cause diarrhea or enteritis. Further results were irritation of

TABLE 1.—*The effect of feeding scabby barley for 10 days on the weights of two lots of hogs*

| Lot | Animals | Weight | | | |
|---|---|---|---|---|---|
| | | Grain | Initial | Final | Gain or loss |
| | | | *Lbs.* | *Lbs.* | *Lbs.* |
| No. 1 | No. 1 | Scabby barley | 102 | 88 | −14 |
| | " 2 | " " | 60 | 52 | − 8 |
| | " 3 | " " | 88 | 71 | −17 |
| | " 4 | " " | 70 | 58 | −12 |
| No. 2 | " 5 | Clean barley | 68 | 68 | 0 |
| | " 6 | " " | 83 | 88 | + 5 |

[4] The author is indebted to Professor Charles Murray, Department of Veterinary Pathology, for assistance in carrying out these tests, which were conducted by the Veterinary Division of the Iowa State College.

the alimentary canal and some kind of intoxication. Sloughing of hoofs or shedding of hair did not take place. The 2 animals held as checks and fed on clean barley appeared healthy and did not lose weight. One of them gained 5 pounds. These results are in line with those reported from Germany by Oppermann and Doenecke (6) and Beller and Wedemann (1) who noted nausea and vomiting, losses in body weight, and some degree of intoxication. Beller and Wedemann considered that the disease-causing agent is to be sought for in the endosperm and the glumes and that it is possibly connected with a volatile substance that hogs can detect.

*Experiments with Chickens.*[5]—While the hog experiments were in progress, three similar feeding trials with chickens were undertaken. The chicks were 2, 6, and 8 weeks old, respectively. No attempt was made to provide a balanced ration. To the standard mash different proportions of barley, naturally infected and artificially infected,[6] were added as indicated in the table. The test lasted for fourteen days during which time the birds were under continuous observation. The results of the experiment are recorded in table 2.

Observations made during the experiments indicate that scabby barley, although not palatable to growing chicks, is not poisonous to them: (1) that, while chicks fed on standard mash alone gained in weight, those fed on rations containing scabby barley failed to gain, or made smaller increases in weight, due in part to voluntary starvation; (2) that artificially infected barley was more injurious than naturally infected barley, and (3) that the

[5] These tests were conducted in cooperation with the Poultry Husbandry Department through the assistance of Professor R. L. Cochran.

[6] Preparation of single-spore cultures: Perithecia were collected from infected kernels and crushed in a clean agate mortar. The ground material was suspended in water. By the dilution-plate method of Keitt (2) monosporic ascospore isolations were made. After 12 to 14 hours at 26° C. the ascospores had germinated and the germ tubes had grown sufficiently long to make possible the selection of single spores which were isolated to potato-agar slants, furnishing monosporic cultures for experimental use.

Preparation of meal cultures: For the feeding tests with the fungus, cultures were made on barley from the previous year's crop (1927), in which the infestation was very slight. The grain looked healthy and only rarely could a grain be found that had the bluish-black perithecia-covered tips.

To prepare the cultures, the barley was first soaked in water for 24 hours; then it was placed in Mason jars. Each jar was filled about three-fourths full, plugged with cotton, covered with Kraft paper caps, and autoclaved for 3 hours at 15 pounds' pressure. Three days afterwards when it was found that sterilization was complete, the contents of the jars were inoculated with the fungus from the agar slants.

The fungus was allowed to grow for 35 days, by which time the barley was completely infected. The jars were then emptied and the material was quickly air-dried by use of an electric fan, after which it was made into a meal in an electric grinder. The meal, which had a wine red color, was stored in clean dry Mason jars in the ice box until it should be used.

TABLE 2.—*Effect of a scabby-barley diet for 14 days upon the weight of chicks*

| Experiment | Lot no. | Number of chicks | Ration | Weight in pounds | | |
|---|---|---|---|---|---|---|
| | | | | Initial | Final | Gain or loss |
| I. Chicks 2 weeks old | 9 | 6 | Half culture scabby wheat; half standard mash | 0.98 | 1.08 | +0.10 |
| | 10 | 6 | Half culture scabby barley; half standard mash | 0.98 | 1.18 | +0.20 |
| | 11 | 6 | Naturally scabby barley | 0.98 | 1.56 | +0.58 |
| | 12 | 6 | Culture scabby barley | 0.98 | 1.10 | +0.12 |
| | 13 | 6 | Check: standard mash | 0.98 | 1.52 | +0.54 |
| II. Chicks 6 weeks old | 1 | 20 | Half naturally scabby barley; half standard mash | 9.76 | 13.64 | +3.88 |
| | 2 | 20 | Three-fourths naturally scabby barley; one-fourth standard mash | 9.60 | 11.24 | +1.64 |
| | 3 | 20 | Check: standard mash | 9.80 | 16.34 | +6.54 |
| III. Chicks 8 weeks old | 4 | 6 | Half culture scabby wheat; half standard mash | 6.26 | 6.90 | +0.64 |
| | 5 | 6 | Half culture scabby barley; half standard mash | 6.00 | 6.74 | +0.74 |
| | 6 | 6 | Naturally scabby barley | 5.94 | 7.22 | +1.28 |
| | 7 | 6 | Three-fourths culture scabby barley; one-fourth standard mash | 5.30 | 5.10 | −0.20 |
| | 8 | 6 | Check: standard mash | 6.20 | 7.84 | +1.64 |

lots receiving the highest percentage of artificially scabby barley seemed to be injured most, as indicated by the loss in weight of the birds and also by the rough and ragged conditions of their plumage.

*Experiments with Guinea Pigs.*—Experiments with guinea pigs were begun in the winter of 1929. It was thought that these animals would respond quickly to the different diets, but the results belied the expectations.

Eleven animals were placed for a period of 15 days on a standard diet, consisting of finely ground yellow corn, alfalfa meal, flour middlings, powdered buttermilk, bone meal, and a small quantity of common salt. After 14 days records were taken of their weights. For the test the feeding was apportioned as follows: Three guinea pigs—standard mash plus clean barley, mixed in equal portions; 2 guinea pigs—standard mash plus naturally infected barley, equal proportions; 4 guinea pigs—standard mash plus artificially infected[7] barley, equal proportions; 2 guinea pigs—standard mash alone (checks). The experiment continued for a period of 25 days. The results are recorded in table 3.

The animals that were fed on barley, either clean or scabby, did not

[7] Artificially infected barley was prepared as in case of the experiment with chickens.

TABLE 3.—*Effect of a diet consisting of standard mash and scabby barley for 25 days on the weight of guinea pigs*

| Lot | Number of animals | Ration | Weight in grams | | Gain or loss in per cent |
|---|---|---|---|---|---|
| | | | Initial | Final | |
| 1 | 3 | Half mash, half clean barley | 3177 | 3098 | − 2.49 |
| 2 | 2 | Half mash, half scabby barley | 2407 | 2034 | − 15.94 |
| 3 | 4 | Half mash, and half artificially infected barley | 4329 | 3807 | − 12.06 |
| 4 | 2 | Standard mash (check) | 2030 | 2094 | + 3.15 |

show any signs of disease during the course of this experiment, but were lively and frisked about like those of the check lots. However, all the animals that received any barley in their rations lost weight. Barley is not relished by guinea pigs and, at least in such high proportions, is not a good diet for them. Animals receiving scabby barley, either naturally or artificially infected, lost more heavily than those receiving clean barley. It was observed that they ate the rations rather reluctantly and much food was left over after each feeding. Possibly the loss in weight was due to this voluntary starvation. Scabby barley apparently did not induce disease even though the animals were forcibly fed.

After this test, the animals were restored to a normal ration consisting of the standard growing mash. They were weighed at the end of 20 days and these weights were recorded as the initial weights for a second test in which the quantity of barley was raised to 75 per cent. This ration, however, was entirely refused by the animals and, after 3 days, to save the animals unnecessary starvation, the experiment was terminated.

## SUMMARY

The 1928 crop of barley in the Midwestern States was injured by a severe epidemic of scab due to *Gibberella saubinetii*, and reports that scabby barley caused disease in hogs and chickens were received by the Iowa Agricultural Experiment Station.

Feeding tests with hogs showed that 40 per cent scabby barley was distasteful, and caused vomiting and slight intoxication. There were no other serious complications, such as diarrhea or enteritis.

Poultry fed on scabby barley did not show disease symptoms, although feeds containing barley were not relished.

Guinea pigs lost in weight when put on a half-and-half barley-stand-

ard-mash diet, and while they lost more when the barley used was scabby, they did not become ill. Clean and scabby barley seemed to be equally distasteful to the guinea pigs.

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# ENZYMES OF THE RHIZOMORPHS OF ARMILLARIA MELLEA

W. M. LANPHERE[1]

(Accepted for publication January 2, 1934)

Although considerable work has been done on the presence of enzymes in the mycelium and sporophore of *Armillaria mellea* (Vahl.) Sacc., the writer has found no record of any having been done on the enzymes of the rhizomorphs. Kohnstamm (3), working with the mycelium and sporophores, reported the presence of diastatic, proteolytic, glucoside-splitting, and cellulose-hydrolyzing enzymes. Schmitz and Zeller (5), in their study of the mycelium, reported the presence of maltase, lactase, sucrase, raffinase, diastase, inulase, cellulase, hemicellulase, emulsin, urease, amidase, trypsin, and erepsin. Gerber (1), demonstrated the presence of rennet in 86 different species of fungi, including *A. mellea*.

## MATERIALS AND METHODS

Material was secured from a shallow well that was curbed with Douglas fir lumber. The rhizomorphs grew out into the water from the curbing until a mat was formed over the surface of the water and to a depth of several feet. These rhizomorphs could be gathered by reaching down from the top of the well; thus, material that was free of foreign matter was obtainable in large quantities. It is not known whether the source of food was the curbing of the well, or other organic material near at hand. Hotson (2) has reported the growth of the rhizomorphs in mines and wells, stating that the growth is very abundant.

Two methods were employed in preparing the rhizomorphs for extraction of the enzymes. The first consisted of drying the material at room temperature by means of an electric fan. The dry rhizomorphs were then ground several times in a meat grinder. A known weight of the ground material was then placed in a flask and 4 times that weight of distilled water was added, with a small amount of chloroform as an antiseptic. The mixture was allowed to stand over night to extract the enzymes. It was then filtered through a Buchner funnel, and the enzymes were precipitated from the filtrate with 3 volumes of 95 per cent alcohol. The precipitated enzymes were then collected on filter papers in a Buchner funnel, and the papers allowed to dry at room temperature. The papers were kept in glass-stoppered bottles in the dark until needed.

The rhizomorphs were so very tough and stringy that considerable material was lost in grinding. It also was found that the material was not so

[1] The writer is indebted to Dr. J. W. Hotson and Dr. G. B. Rigg for advice and helpful criticism in conducting the work reported in this paper.

finely ground as was desired. In order to overcome these difficulties, a second method was employed, essentially the same as the first, except that the rhizomorphs were ground in a mortar while they were still fresh. Before grinding, a little sand and distilled water were added to facilitate the process. After the mass had been ground to a fine pulp, enough distilled water was added to bring the water content up to approximately 4 times the weight of the rhizomorphs, had they been dried. The remainder of the process is identical with that described above.

When the enzyme material was to be used, the filter papers were soaked in a quantity of water so that 1 cc. of the resulting enzyme solution would be equal to one gram of the fresh rhizomorphs. For instance, 10 grams of material fresh from the well would, when precipitated on the filter papers, require 10 cc. of distilled water at this stage of the procedure.

All experiments, unless otherwise described, were set up in the following manner: 10 cc. of the prepared substrate was put into each of 3 test tubes. Two cc. of the enzyme solution was added to the first tube; to the second, was added 2 cc. of the enzyme solution that had been autoclaved at 10 pounds' pressure for 10 minutes; to the third tube was added 2 cc. of distilled water.

If the results of the experiment were positive, the first tube would show positive results, while the second and third tubes, the controls, would show negative results. Toluol was used as the preservative in all experiments that were allowed to run for more than a few hours.

TABLE 1.—*Results of experiments conducted to detect the presence of enzymes in the rhizomorphs of Armillaria mellea*

| Test for | Substrate | Incubation period | Result |
|---|---|---|---|
| Diastase | Potato starch | 4 hours | + |
| Inulase | Inulin | 3 days | + |
| Hemicellulase | Date endosperm | | |
| | Van Tieghem cells | 45 " | – |
| | Test-tube method | 30 " | – |
| Invertase | Sucrose | 1 day | + |
| Maltase | Maltose | 25 days | – |
| Lactase | Lactose | 25 " | – |
| Rennet | Milk | 36 hours | + |
| Oxidase | Guaiacum | 2 " | + |
| Peroxidase | " | 2 " | + |
| Catalase | Hydrogen peroxide | | + |
| Esterases | Olive-oil emulsion | 25 days | – |
| " | Ethyl acetate | 25 " | – |

TESTS OF THE RHIZOMORPHS OF ARMILLARIA MELLEA FOR ENZYMES

*Diastase.*—Starch paste was used as the substrate. Potato starch was converted into a 0.25 per cent paste according to the following method: Two and one-half grams of potato starch in 150 grams of distilled water were brought to a boil while constantly stirred. It was then transferred to a flask containing about 600 cc. of hot distilled water. The whole was boiled in a reflux condenser for 2 hours. After cooling, the volume of the paste was made up to a liter by adding distilled water. One per cent toluol was added and the flask plugged until ready for use.

The experiment was set up as described above. The Fehling's test was applied after 4 hours.

*Inulase.*—A 1 per cent solution of inulin was used as the substrate. The experiment was set up as described in the foregoing. The tubes were incubated at 30° C. After 3 days the Fehling's test was applied.

*Hemicellulase.*—For this experiment, the endosperm of the date seed was used as the source of the hemicellulose. Date seeds were scraped to remove the outer coat. They were then scrubbed with sand and soap in order to remove all the reducing sugars possible, washed in distilled water, cracked, and the embryos removed. The endosperms were then autoclaved in distilled water at 15 pounds' pressure for ½ hour in order to destroy any enzymes that might be present. They were then rinsed and stored in distilled water until needed, with toluol as the preservative.

Two methods of testing for hemicellulase were employed. Very thin slices of the date endosperm were cut on the microtome and suspended in hanging drops in properly prepared van Tieghem cells. Cells were set up as follows: (1) Three cells with date endosperm suspended in a drop of enzyme solution; (2) three cells with date endosperm suspended in a drop of enzyme solution that had been autoclaved; and (3) three cells with slices of the date endosperm suspended in distilled water. Enough of the solution was placed in the bottom of each cell to prevent evaporation. Chloroform was used as an antiseptic. The cells were incubated at 30° C. and were observed from time to time for signs of erosion of the endosperm. Although the cells were observed over a period of 45 days, no erosion was detected.

The second method consisted of placing pieces of the date endosperm in each of 3 test tubes. To the first was added 10 cc. of the enzyme solution; to the second was added 10 cc. of the enzyme solution that had been autoclaved; and to the third was added 10 cc. of distilled water. Toluol was used as the antiseptic. The tubes were incubated for 30 days at 30° C., they were then tested for reducing sugars with Fehling's solution. Results were negative.

*Invertase.*—A 1 per cent solution of sucrose was used as the substrate. Tubes were set up as described above. They were allowed to incubate at 30° C. for 24 hours, then tested with Fehling's solution.

*Maltase.*—A 1 per cent solution of maltose was used as the substrate. Tubes were set up as described above and allowed to incubate at 30° C. for 25 days. They were then removed from the incubator and the Fehling's test applied. Since maltose reduces Fehling's solution, it was necessary to apply some quantitative test in order to determine the presence of the enzyme. One molecule of maltose is hydrolyzed into 2 molecules of glucose. From this fact it is possible to determine the presence of the enzyme by an increase in the amount of Fehling's solution reduced. The following method was used in determining the amount of Fehling's solution reduced: After applying the Fehling's test, filter through a Gooch filter, dissolve the copper oxide with 5 cc. of 6N nitric acid, wash with 10 cc. of distilled water, boil to expel red fumes, dilute to 25 cc., add 3N NaOH to permanent turbidity, clear the solution with 6N acetic acid, plus 1 cc. excess, dilute to 40 cc. with distilled water. Add potassium iodide crystals, titrate with sodium thiosulphate, using fresh starch solution as the indicator.

*Lactase.*—A 1 per cent solution of lactose was used as the substrate. The procedure was the same as for maltase.

*Rennet.*—Fresh cow's milk was used as the substrate. The experiment was set up as described above. The tubes were incubated at 30° C. for 24 hours, whereupon the first tube showed distinct curds, while the controls showed none. After 36 hours, the first tube was completely coagulated, while the controls showed no coagulation.

*Oxidase.*—In testing for oxidase, a 10 per cent solution of guaiacum gum was used. Since the gum oxidizes in the air, large pieces were selected. The outside of the pieces was cut off with a sharp knife so that only the interior of the lumps was used. The gum was dissolved in alcohol. Five cc. of the enzyme solution was placed in a test tube; in a second tube, 5 cc. of the boiled enzyme solution was placed. A few drops of the guaiacum solution was added to each tube. After two hours, the first tube showed the blue color, indicating the presence of an oxidase. The control showed no color for twelve hours.

*Peroxidase.*—The experiment was set up exactly as was the oxidase experiment. On adding the guaiacum-gum solution, no color developed, but on the addition of a few drops of ordinary hydrogen peroxide, a blue tint began to appear. In 15 minutes, a deep blue had developed in the first tube. The control showed no color even after standing for several hours.

*Catalase.*—When a few drops of hydrogen peroxide were added to 2 cc. of the enzyme solution, bubbles were formed in great abundance. When

this was repeated with the boiled enzyme solution, no oxygen was given off, thus indicating the presence of catalase.

*Esterases.*—Experiments were conducted using olive-oil emulsion and ethyl acetate as substrates. Olive-oil emulsion was made according to the method of Bloor. Ten cc. of olive oil was dissolved in hot absolute alcohol. This solution was run through a hot funnel, to which was attached a piece of glass tubing drawn out to a fine jet, into 100 cc. of constantly stirred, cold, distilled water. The milk-white emulsion was boiled to drive off the alcohol, and was diluted to 500 cc. with distilled water.

For the ethyl acetate experiment, a 1 per cent solution of ethyl acetate was used as the substrate.

Experiments were set up as described in the first part of this paper. The tubes were incubated for 25 days at 30° C. The contents of the tubes were then titrated with N/20 NaOH, using phenolphthalein as the indicator.

## DISCUSSION

The list of enzymes for which tests were made is far from complete. The fact that negative results were obtained in some cases does not prove that the enzyme does not occur in the rhizomorphs. When positive tests were secured, they were repeated until there could be no doubt of the presence of the enzyme. It is not possible from the results so far obtained to make any positive statement as to the rôle played by the enzymes found in the rhizomorphs. Whether they are concerned with the entry of the rhizomorphs into the host was not determined, though it seems that such may be possible. Thomas (6), however, is of the opinion that the entry is largely due to mechanical pressure.

## SUMMARY

The enzymes found in the rhizomorphs of *Armillaria mellea* are diastase, inulase, invertase, rennet, oxidase, peroxidase, and catalase; the enzymes for which tests were made and not found are hemicellulase, maltase, lactase, and the esterases.

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# INOCULATION TESTS WITH VERTICILLIUM WILT OF MUSKMELONS[1]

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(Accepted for publication January 8, 1934)

In late July, 1932, a serious wilt occurred in a planting of the Persian variety of muskmelons, *Cucumis melo* L., in San Joaquin County, California. The first evidence of the disease was the wilting of the crown leaves just before harvest, followed very soon by the wilting and death of the entire plant. Additional diseased plants continued to appear over a period of several weeks, with the resultant death of 15 to 20 per cent of the stand. These plants showed a brown discoloration in the vascular tissues of the root, stem, and large lateral shoots, and freehand sections showed the presence of mycelium in the discolored regions. Repeated isolations from the roots and stems of diseased plants yielded mostly pure cultures of a species of Verticillium.

The Persian melons were growing in the middle section of the field and on one side of this section, the Honey Dew variety of muskmelons, *C. melo* L. var. *inodorus* Naud., was growing, while the variety, Hale's Best, *C. melo* L. var. *reticulatus* Naud., was growing on the other side. No evidence of the disease showed in either the Honey Dew or Hale's Best plantings. The marked susceptibility of the Persian variety has been reported by Rudolph[3].

The pathogenicity of the Verticillium isolated, identified by B. A. Rudolph as *V. albo-atrum,* was established in the fall of 1932 by growing Persian melons in 6-inch pots of steam-sterilized soil to which a heavy water suspension of Verticillium spores was added so as to wet the top to a depth of 2 inches. Additional successful inoculations were secured by placing fragments of agar from a culture of Verticillium in contact with wounded roots. The results, however, obtained by growing plants in infested soil, showed that wounding the roots was not necessary to produce the disease.

Under greenhouse conditions, the first symptoms of the disease were evident in approximately 6 weeks from date of planting and were manifest by a slight yellowing of the leaves near the crown and wilting and curling of irregular areas along the margin of the yellowed leaves. There was a somewhat slower progression of the wilt symptoms from the first foliage leaves to the growing tips of the vines than under field conditions, but eventually

[1] Contribution from the Division of Plant Pathology, Branch of the College of Agriculture, University of California, Davis, California.

[2] The writers are indebted to Professors Ralph E. Smith and Max W. Gardner for suggestions and criticisms.

[3] Rudolph, B. A. Verticillium hadromycosis. Hilgardia **5**: 197–353. 1931.

the entire plant wilted and died. Diseased plants showed the characteristic vascular discoloration in the roots, stem, and branches, especially in the axillary leaf-petiole and branch tissue. In a critical study of 6 diseased plants, resulting from artificial inoculation, the causal fungus was recovered from all parts of the main shoots up to within a few inches of the growing tips.

Reference to the literature shows that Van der Lek[4] found a Verticillium associated with a melon disease in Europe as early as 1918. Gram and Rostrup,[5] in 1925, and Dufrénoy,[6] in 1927, also report the occurrence of a Verticillium disease of melons, but none of these workers gives any account of pathogenicity studies. Bewley[7] (pp. 83–84) isolated the fungus from melons and certain other hosts and obtained successful inoculations. In America, Rudolph (*loc. cit.*) isolated from diseased muskmelon plants a species of Verticillium that he considered to be *V. albo-atrum*.

The occurrence of the disease in a severe form on Persian melons in 1932 in a field where Hale's Best and Honey Dew varieties were growing without showing evidence of the disease, would lead one to suspect that these 2 varieties were possibly resistant to the fungus. Controlled greenhouse trials were carried out to determine the relative susceptibility of the common commercial varieties of muskmelons grown in California. Cultures of Verticillium growing on steamed oats were uniformly mixed into a large quantity of steam-sterilized soil with which 6-inch pots were filled. On February 21, 1933, 4 series of 16 pots each were planted with seed of 4 of the principal varieties grown in California, Hale's Best, Honey Ball (*Cucumis melo* L. var. *inodorus* Naud.) Honey Dew, and Persian. An additional series was started on March 7, consisting of 35 pots of the Casaba variety *C. melo* L. var. *inodorus* Naud., 16 pots of Hale's Best, 18 pots of Honey Ball, 20 pots of Honey Dew, and 18 pots of Persian. When the plants were putting out their first foliage leaves they were thinned to 4 plants per pot. In 6 weeks from date of planting, the first symptoms of wilting were evident and were comparable in every way with those secured in the earlier pathogenicity studies. For the records, which were taken on May 12 and June 1, each plant was examined carefully for wilt symptoms by cutting the stems and culturing from diseased plants. The results of the above tests are presented in table 1.

[4] Van der Lek, H. A. A. Onderzoekingen over tracheomycosen: De verticilliose van den komkommer. Med. Landbouwhoogesch. Wageningen **15**: 1–45. 1918.

[5] Gram, E., and S. Rostrup. Oversigt over sygdomme hos landbrugets og havebrugets kulturplanteri, 1924. Tidsskr. Planteavl **31**: 353–417. 1925.

[6] Dufrénoy, J. Déperissement des arbres fruitiers dans le Massif Central. Bul. Off. Agr. Rég. Massif Central Clermont-Ferraud **7**: 103–113. 1927.

[7] Bewley, W. F. Diseases of glasshouse plants. 208 pp. E. Benn, limited, London. 1923.

TABLE 1.—*Susceptibility tests of 5 commercial muskmelon varieties, commonly grown in California, to Verticillium wilt under greenhouse conditions*

| Variety | Total number of plants | Number dead or diseased | Percentage diseased |
|---|---|---|---|
| Casaba | 137 | 126 | 91.7 |
| Hale's Best | 154 | 120 | 77.8 |
| Honey Ball | 124 | 67 | 54.0 |
| Honey Dew | 150 | 122 | 81.3 |
| Persian | 135 | 119 | 88.1 |

The results of susceptibility trials presented in table 1 show that all of the 5 main crop-melon varieties grown in California, including Hale's Best and Honey Dew, are susceptible to Verticillium wilt and, with the exception of Honey Ball, no material difference in susceptibility was evident. Honey Ball, while showing considerably less disease than the other 4 varieties, was in no sense immune.

## SUMMARY

A destructive Verticillium wilt occurred in a large planting of Persian muskmelons in California in 1932. The varieties Hale's Best and Honey Dew, growing in the same field, did not show the disease. Greenhouse tests established the pathogenicity of the Verticillium in question and also showed that, under greenhouse conditions, the varieties Casaba, Hale's Best, Honey Ball, Honey Dew and Persian are susceptible to the disease.

# BIG VEIN, A DISEASE OF LETTUCE

IVAN C. JAGGER AND NORMAN CHANDLER

(Accepted for publication March 14, 1934)

In 1922 an investigation of brown blight of lettuce[1] was begun in the Imperial Valley, California. This disease causes a pronounced stunting and gradual dying of plants, accompanied by characteristic yellow, discolored spots in younger leaves and brown, dead, irregular blotches and streaks in older ones. From the first, and often associated with characteristic brown blighted plants, occasional plants were observed that showed very striking, enlarged light yellow leaf veins (Fig. 1, A). It was at first thought that this striking symptom might be another phase of brown blight, but it now seems probable that big vein is a disease wholly distinct from brown blight.

Big vein is not apparent on lettuce until after the seedlings have 5 or 6 leaves, when plants are attacked in all stages of growth. The first noticeable symptom is a slight yellowing along the veins. This gradually becomes more pronounced, accompanied by considerable thickening of the whole leaf and more or less crinkling, until the pronounced symptoms shown in figure 1 are evident. All leaves of affected plants show symptoms more or less characteristic of a systemic disease. There are never any indications of plants being killed or of dead or brown tissue in any part of the plants. Symptoms are most pronounced in actively growing plants sometime before maturity. When the heads have reached maximum firmness and are ready for harvesting, the symptoms usually are less pronounced, for the yellow veins become somewhat green and there is a normal fading of interlying green leaf areas at maturity. In the winter-lettuce sections, in crops maturing during the cooler weather of midwinter, big-vein symptoms are usually more pronounced, and there are possibly higher percentages of affected plants than in crops maturing during the relatively warmer weather of late fall and early winter. Affected plants continue to grow and to head, but the heads are reduced to about half size and are of doubtful quality and value.

There has been a slow increase in the occurrence of big vein in the Imperial Valley from the time it was first observed. About 1929 it began to cause appreciable injury in a few fields and to attract the attention of growers. During the past few years it has become necessary to discontinue

[1] Jagger, Ivan C. Lettuce breeding for disease resistance progresses rapidly. U. S. Dept. Agr. Yearbook **1931**: 348–350. 1931.

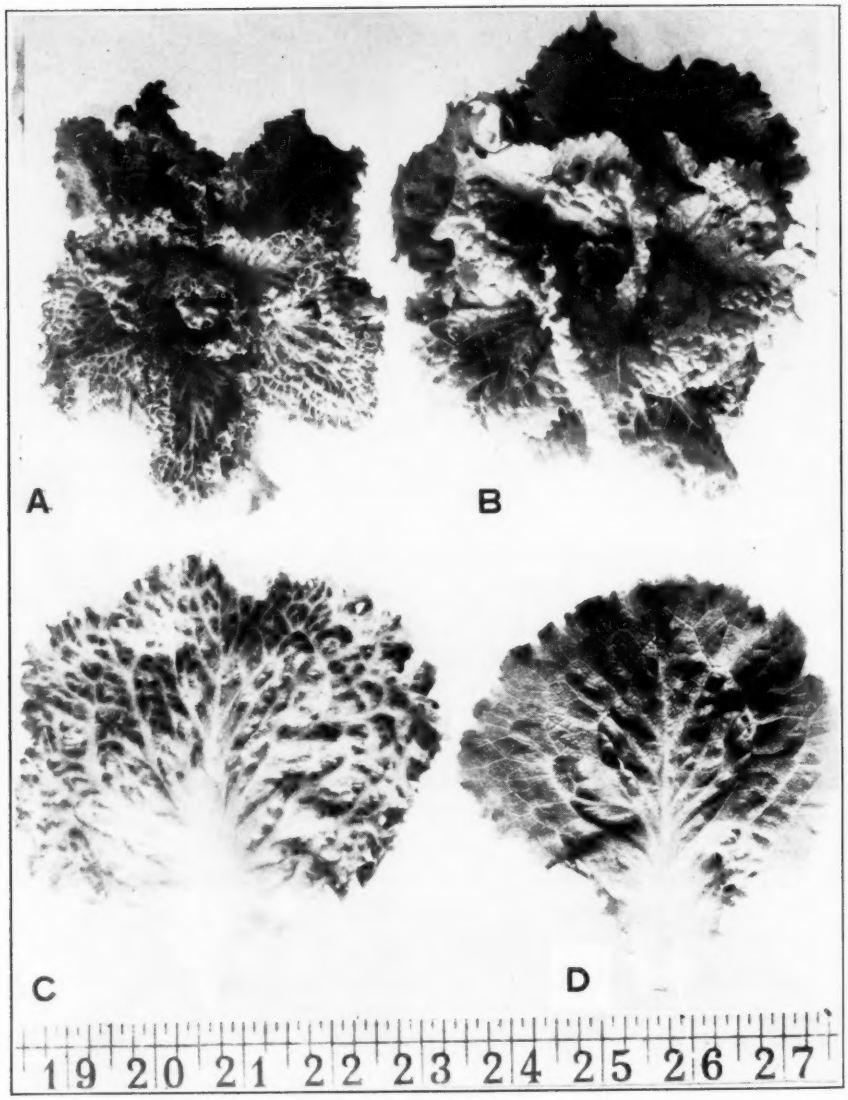


FIG. 1. Iceberg type lettuce. A. Young plant affected with big vein. B. Healthy young plant. C. Leaf affected with big vein. D. Healthy leaf.

lettuce on a very limited acreage of soil where several consecutive crops of it have been grown and upon which lettuce has become seriously affected. But the disease is not yet seriously interfering with the lettuce industry of the Valley as a whole. For several years occasional typical big-vein specimens have been found in the Salinas-Watsonville lettuce section and in

other smaller sections in that part of California. Although there is probably a slow increase in the disease from year to year, no commercial injury to crops has so far been recorded in those sections. During the past 2 or 3 seasons it has been noted in the principal lettuce sections of Arizona. The disease is largely confined at present to heavier, naturally poorly drained soils, and, so far as known, it occurs only in California and Arizona.

Big vein is a soil-borne disease, which increases from year to year in favorable types of soil under constant lettuce culture. In pot cultures it has never attacked plants in containers of noninfested soil, although surrounded with containers of infested soil in which all plants showed big veins. New lettuce land, separated from old, severely infested lettuce land by only a roadway or a ditch, usually shows only a trace of the disease the first year. Big vein is apparently not readily transmitted except through soil. The application of various chemicals and fertilizers to infested soil, such as manganese sulphate, zinc sulphate, boric acid, iron sulphate, copper sulphate, various commercial fertilizers, stable manure and green cover crops, has not reduced the disease. In pot cultures a considerable percentage of the soluble salts have been leached out of infested soils without affecting the disease. Partial sterilization of the soil with either steam or formaldehyde eliminates it. Symptoms and behavior of big vein indicate that it may be similar to the soil-borne mosaic of wheat,[2] although no definite proof of its being caused by a virus has yet been obtained.

At present the only method of avoiding losses from big vein is to discontinue lettuce on land that has become sufficiently infested to cause appreciable injury to the crop. Thus far, it has become destructive only where several crops of lettuce have been grown in succession, and it is possible that a relatively short rotation with other crops may control it. Definite information on this point, however, is not yet available. Considerable effort has been made to obtain varieties or strains of lettuce that are resistant to the disease, but very little progress has been made.

Big vein and brown blight have the same general distribution, but in several smaller lettuce areas only one or the other has been found. In areas where both occur many fields show both, but in others either may occur alone. The occurrence of brown blight is related to the same soil conditions as described above for big vein, and it is possible that the two diseases are of similar nature. Brown blight, however, increases in severity and spreads much more rapidly than big vein, on a wide range of soil types and without responding readily to control by crop rotation. Brown blight had attained alarming prevalence and severity, threatening the lettuce industry of California and Arizona with destruction prior to its being brought under com-

[2] McKinney, H. H. A mosaic disease of winter wheat and winter rye. U. S. Dept. Agr. Bull. 1361. 1925.

plete control by essentially immune strains of lettuce that have been developed by the writers during the past few years. Before brown-blight-resistant strains were available, much soil in California and Arizona became so severely infested with the disease after producing 2 or 3 crops of lettuce that it was necessary to discontinue its culture. Under such conditions the less virulent big vein seldom became prevalent, but now the growing of brown-blight-resistant strains for several years without rotation apparently is causing certain [permitting favorable] soils to become heavily infested with the big-vein disease. In every way big vein appears to be a less serious disease than brown blight, but it may become generally troublesome, at least on the heavy soils of California and Arizona, if definite methods of control are not found.

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# INHERITANCE OF RESISTANCE TO POWDERY MILDEW, ERYSIPHE GRAMINIS TRITICI, IN WHEAT

E. B. MAINS[1]

(Accepted for publication January 11, 1934)

In a previous publication,[2] it has been shown that a number of varieties of wheat possess more or less resistance to the *tritici* race of *Erysiphe graminis* DC. The varieties Axminister (C.I. 1839),[3] Chul (C.I. 2227), Dixon (C.I. 6295), Huron (C.I. 3315), and several selections of Illinois No. 1, Norka (C.I. 4377), Red Fern, and Sonora (C.I. 4293) were outstanding among common wheats for their marked resistance to physiologic form 1. To this physiologic form of the mildew they showed few or no signs of infection in the seedling stage when inoculated with conidia. All except Norka and Axminister were similarly highly resistant to physiologic form 2. In marked contrast they showed pronounced susceptibility to physiologic form 2.

## RESISTANCE-INHERITANCE STUDIES

In 1928, attention was first directed to a study of the inheritance of resistance. Crosses were made between a number of the resistant varieties and various susceptibility wheats in 1928, 1929, and 1930. The first generations were grown in the field. The second generations were for the most part planted in the greenhouse and inoculated in the seedling stage (3–4 leaf). This was easily and thoroughly accomplished by developing the mildew on a susceptible variety. When the mildew was sporulating abundantly, pots of the infected plants were shaken over the seedlings to be studied. These were then atomized with a fine spray of water and covered for 24 hours with wet muslin cloths. The resulting infection was abundant and usually uniform. In 10 to 14 days, the infection had reached a development so that seedlings could be classified for their mildew reaction. In most cases inoculated seedlings were not grown to maturity. In instances where the third generation was studied a portion of the second generation was spaced in the field and each plant harvested separately. about 15–25 seedlings of each of the lines of the third generation were then inoculated and studied in the greenhouse.

[1] This study was started while the writer was a member of the Department of Botany, Purdue Agricultural Experiment Station, and of the Division of Cereal Crops and Diseases of the United States Department of Agriculture. It has been continued at the University of Michigan. Papers of the Department of Botany and the Herbarium of the University of Michigan. No. 458.

[2] MAINS E. B. Host specialization of *Erysiphe graminis tritici*. Proc. Nat. Acad. Sci. 19: 49–53. 1933.

[3] Accession numbers of the Division of Cereal Crops and Diseases.

1. *Inheritance of Resistance of Norka to Mildew.* Until physiologic form 2 of the mildew was discovered in 1932, Norka was one of the most outstanding varieties for resistance. Throughout 5 years of previous study it showed no signs of infection beyond occasional faint flecking, although repeatedly inoculated.

For this study Norka was crossed with Webster (C.I. 3780), Ceres (C.I. 6900), Chinese (C.I. 6223), Kota (C.I. 5878), Malakoff (C.I. 4898), Reliance (C.I. 7370), and Warden (C.I. 4994), varieties that were found to be very susceptible to powdery mildew in the seedling stage. In the second generation the progenies segregated into 2 very distinct classes. One was highly susceptible like the susceptible parent. In marked contrast, the other showed little or no evidence of infection. While most of the seedlings of this group were fully as resistant as the Norka parent, some showed a very slight development of mildew. Table 1 gives the results obtained from a study of the second generation of these crosses.

It is evident in all of the crosses that the resistance of Norka segregated in a ratio closely approximating 3 resistant to 1 susceptible.

Two of the crosses were studied in the third generation. In the cross Norka × Chinese, 662 $F_3$ lines were inoculated. Of these 160 proved to be uniformly resistant, 349 contained resistant and susceptible individuals, and 153 were uniformly susceptible. This is a very close approximation to the 1:2:1 ratio of 165.5:331:165.5 expected, on the basis of a single genetical factor. The 349 segregating lines gave a total of 14,062 individuals. Of these 10,563 were resistant and 3,499 susceptible, giving a ratio of 3.005:.995.

TABLE 1.—*Inheritance of the resistance of Norka to Erysiphe graminis tritici p.f.1. Segregation in $F_2$*

| Susceptible parent | Resistant segregates | Susceptible segregates | Ratio | Dev. | P.E. | $\frac{\text{P.E.}}{\text{Dev.}}$ |
|---|---|---|---|---|---|---|
| Webster | 654 | 213 | 3.02:0.98 | 3.75 | 8.60 | 0.44 |
| Ceres | 1346 | 488 | 2.94:1.06 | 29.50 | 12.50 | 2.36 |
| Chinese | 446 | 158 | 2.96:1.04 | 7.00 | 7.18 | 0.97 |
| Kota | 133 | 50 | 2.91:1.09 | 4.25 | 3.95 | 1.09 |
| Malakoff | 592 | 191 | 3.03:0.97 | 4.75 | 8.17 | 0.58 |
| Reliance | 117 | 41 | 2.96:1.04 | 1.50 | 3.67 | 0.41 |
| Warden | 1173 | 366 | 2.95:1.05 | 18.75 | 11.46 | 1.64 |

In the cross Norka × Reliance, 136 $F_3$ lines were inoculated. Of these 29 were uniformly susceptible, 74 segregated into resistant and susceptible groups, and 33 were uniformly susceptible. This is a very close approximation to the expected 1:2:1 ratio of 34:68:34. In the 74 lines segre-

gating for resistance and susceptibility, a total of 2,729 individuals were studied. Of these 2,022 were resistant and 707 were susceptible, giving a ratio of 2.96 : 1.04.

It is evident from the data presented that the resistance of Norka to physiologic form 1 is inherited as a simple dominant Mendelian factor.

2. *Inheritance of Resistance of Norka to Mildew in Relation to Inheritance of Resistance to Leaf Rust.* Norka also has shown pronounced resistance to a number of the physiologic forms of the leaf rust of wheat *Puccinia rubigo-vera tritici* (*Puccinia triticina* Eriks.).[4] Since Norka is highly resistant to physiologic form 3 of this rust, the second generation of the cross Norka × Ceres was studied for inheritance of the resistance of Norka to both p.f.1 of mildew and p.f.3 of leaf rust, Ceres being susceptible to both. This was done by inoculating with both mildew and rust simultaneously. Plants susceptible to the rust produced uredinia, while those resistant showed only faint flecking or occasionally small uredinia.

A little difficulty was experienced in classifying the reaction of some of the plants to rust due to the peculiar effect of mildew on rust reaction. As had been noted a number of times previously in the leaf-rust investigations, when a variety of wheat highly resistant to leaf rust became infected with both mildew and rust rather large uredinia of the rust would develop in places where the mycelia of the two fungi intermixed.[5] In portions of the leaf tissue where the mycelia of the mildew were not present no uredinia and only flecks developed. Apparently, the cells of the host invaded by the mildew were more favorable for the development of the mycelia of the rust than uninfected cells. Although this makes some difficulty in classifying the rust-resistant individuals, by careful examination of such plants in areas uninfected by the mildew they can be properly placed.

Out of 1,834 individuals studied for reaction to physiologic form 3 of leaf rust 1,409 were resistant and 425 susceptible. This gives a ratio of 3.07 : 0.93, closely approximating a 3 : 1 ratio. The deviation is 33.5 and P.E. 12.50, the deviation ÷ P.E. equaling 2.68. This indicates that resistance of Norka to p.f.3 of leaf rust is due to a simple Mendelian factor. According to their reactions to both rust and mildew the individuals were classified into four groups. Of the 1,834 plants, 106 were susceptible to both rust and mildew, 319 were susceptible to rust and resistant to mildew, 382 were resistant to rust and susceptible to mildew, and 1,027 were resistant to both rust and mildew. This gives a ratio of .92 : 2.78 : 3.33 : 8.97. These results agree closely with those expected for the segregation of 2 independent factors.

[4] MAINS, E. B., and H. S. JACKSON. Physiologic specialization in the leaf rust of wheat, *Puccinia triticina* Eriks. Phytopath. **16**: 89–120. 1926.

[5] See also discussion of this phenomenon by Johnston, C. O., Phytopath. **24**: 1045–1046. 1934.

3. *Inheritance of Resistance of Red Fern to Mildew.* Red Fern has shown a very high resistance to both physiologic forms of powdery mildew throughout the 6 years of study. This variety was crossed with the susceptible Chinese (C.I. 6223), and 3,333 plants of the second generation were studied. These were separated into a resistant group giving a reaction similar to the Red Fern parent and a susceptible group having a reaction similar to Chinese. The segregation was 2,508 resistant to 825 susceptible, giving a ratio of 3.01 : .99 a Dev. of 8.25, P.E. 16.87. The resistance of Red Fern is, therefore, also due to a simple dominant Mendelian factor.

4. *Inheritance of Resistance of Hope (C.I. 8178).* The resistance of Hope to physiologic form 1 was somewhat more difficult to study than that of the previous varieties discussed. Its reaction is more or less variable. It usually gives a 0 to 1 reaction, but occasionally may reach about 2+. The most resistant reaction was produced during midwinter and the least in late spring. In order to study segregation with any degree of accuracy it is desirable to make the inoculations during midwinter. Even so the separation into classes in this cross was much more difficult than in those with Norka and Red Fern. Plants showing reactions of 0–2 were classed as resistant and those having reactions of 3–4 were classed as susceptible. The results of these studies are given in table 2.

TABLE 2.—*Inheritance of the resistance of Hope to Erysiphe graminis tritici p.f.1. Segregation in* $F_2$

| Susceptible parent | Resistant segregates | Susceptible segregates | Ratio | Dev. | P.E. | $\frac{\text{Dev.}}{\text{P.E.}}$ |
|---|---|---|---|---|---|---|
| Chinese | 89 | 302 | 0.91 : 3.09 | 8.75 | 5.78 | 1.51 |
| Marquis | 45 | 112 | 1.15 : 2.85 | 5.75 | 3.66 | 1.57 |
| Michigan Amber | 83 | 287 | 0.90 : 3.10 | 9.50 | 5.62 | 1.69 |
| Reliance | 125 | 420 | 0.92 : 3.08 | 11.25 | 6.82 | 1.65 |

The third generation was studied in the case of 2 crosses. In the cross Hope × Chinese, 205 $F_3$ lines were studied. Of these, 51 proved to be uniformly resistant, 106 showed segregation, and 48 were uniformly susceptible. This is very close to the expected 1 : 2 : 1 ratio, which would be 51 : 102 : 51. The 106 segregating lines produced 868 resistant and 2,515 susceptible individuals, a ratio of 1.03 : 2.97.

For the cross Hope × Marquis 236 $F_3$ lines were studied. Of these, 59 were uniformly resistant, 122 segregated and 55 were uniformly susceptible. This also is very close to the expected 1 : 2 : 1 ratio which would be 59 : 118 : 59. The 122 segregating lines produced a total of 1,315 resistant

individuals and 3,636 susceptible, giving a ratio of 1.06 : 2.94. Apparently, the resistance of Hope to physiologic form 1 is due to a single recessive factor.

5. *Inheritance of Resistance of Sonora.* The resistance of Sonora to physiologic form 1 was studied in a cross with Chinese. Of the 131 plants of the $F_2$, 38 were highly resistant and 93 were very susceptible. This is a ratio of 1.16 : 2.84 with a Dev. of 5.25 and P.E. of 3.34. Apparently, the resistance of Sonora is recessive and also due to a single factor.

6. *Inheritance of Resistance of Michigan Amber 29–1–1–1.* The reaction of Michigan Amber 29–1–1–1 to p.f.1 is very unusual. Of all the varieties studied, it has shown the greatest variability. In the seedling stage in the greenhouse, during the winter months, it was usually 0 to 1. Under more favorable growing conditions for the variety it was much more susceptible, even giving a reaction of 3+. When this reaction was first encountered early in the fall, it was very striking in contrast with the marked resistance previously noted for the variety, and it was thought that a new physiological form had been found. Inoculations of Michigan Amber 29–1–1–1, later in the season, showed that it was a variation in the reaction of the variety. This condition made a study of the inheritance of the resistance especially difficult. The first attempt made late in the winter showed no sharp separation into classes, so that no conclusions were possible. However, a population of 297 plants of the $F_2$ of a cross between Michigan Amber 29–1–1–1 and Chinese (C.I. 6223) was studied earlier the succeeding winter when Michigan Amber was showing pronounced resistance. This segregated into 81 resistant (0–2) and 216 susceptible (3–4), a ratio of 1.09 : 2.91 with a Dev. of 6.75 and P.E. of 5.03. This would indicate that the resistance manifested in the seedling stage is probably inherited as a recessive simple factor. Further study with the variety would seem to be desirable.

## SUMMARY

The resistance of Norka (C.I. 4377) in the seedling stage to physiologic form 1 of *Erysiphe graminis tritici* is inherited as a simple dominant factor. The same is true for the resistance of Red Fern.

The resistance of Hope (C.I. 8178) in the seedling stage is inherited as a simple recessive factor. The same is true for the resistance of Sonora (C.I. 4293) and apparently also for Michigan Amber 29–1–1–1 under conditions favorable for its maximum expression.

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# THE RESISTANCE OF SAPLINGS AND CERTAIN SEEDLINGS OF PINUS PALUSTRIS TO SEPTORIA ACICOLA[1]

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Before vigorous height growth has started, most seedlings of *Pinus palustris* Mill. are very susceptible to attack by *Septoria acicola* (Thum.) Sacc., the pathogen of the brown-spot needle blight,[2,3] the fungus readily advancing in the needles to cause large dead areas by the end of the first season of the needles. During the spring of the second season the advance is so fast in severely infected seedlings, especially when grass and other ground cover are scarce around the seedlings, that typical brown spots are not formed but whole needles yellow and die below the old brown spots. After vigorous height growth starts, and with certain individual seedlings before it starts, a marked resistance to the fungus is evident, the brown spots being small and definitely limited, even through the second growing season of the needles.

Associated with typical brown-spot lesions on resistant seedlings, saplings, and to a less extent on mature trees, are "bar-spots," consisting of a small area surrounded by a yellow zone that may extend from less than 1 to 3 mm. from the brown area. Bar-spots have but a sparse mycelium in the mesophyll layer, have many resin-filled cells, and only very rarely possess the fruit bodies of Septoria. They seem to indicate resistance, the host reacting to prevent the normal growth and reproduction of the fungus. Bar-spots have been found on *Pinus caribaea, P. pinaster, P. glabra, P. taeda,* and *P. echinata,* in addition to *P. palustris.*

During the period of January to July, 1933, a number of observations and experiments were made near Bogalusa, Louisiana, to determine the nature of this resistance.

Saplings 6 to 10 feet high, bent over so that the terminal buds opened within a few inches of abundantly infected seedlings, developed only 0.5 per cent dead leaf length by June 23, while the near by seedlings showed about 15 per cent at the same time, indicating that the resistance of sap-

[1] The investigations here reported were made by the writer while employed as Field Aid by the Division of Forest Pathology, Bureau of Plant Industry, in cooperation with the Southern Forest Experiment Station, U. S. Forest Service. Acknowledgment is due Mr. P. V. Siggers of the Division of Forest Pathology for much assistance.

[2] Hedgcock, Geo. G. *Septoria acicola* and the brown-spot disease of pine needles. Phytopath. 19: 993–999. 1929.

[3] Siggers, Paul V. The brown-spot needle blight of longleaf pine seedlings. Jour. For. 30: 579–593. 1932.

lings is real. There seems to be a change from susceptibility to resistance with the initiation of vigorous height growth.

The resistance of certain seedlings is likewise real. An almost disease-free seedling may be growing beside one with a high degree of infection. Both may be growing under the same conditions of soil and ground cover, and the susceptible plant may be smaller or larger and with fewer or more leaf fascicles than the diseased one.

The anatomical differences between the leaves of saplings and seedlings and of resistant and susceptible seedlings are, in general, independent of the amount of dead leaf length and are determined chiefly by the degree of shade in which the leaves developed. Stomata are sufficiently abundant on all to insure avenues of entrance for the pathogen. However, seedlings often times have but 2 resin ducts in a needle, while saplings usually have 3 or 4. Susceptible and resistant seedlings do not differ from each other in this respect.

The difference between the number of resin ducts of saplings and seedlings suggested that resin production may have some bearing on resistance. Diseased spots were dissected from needles and the resin extracted with an excess of ether. The samples were dried for 2 hours at 100° C. and weighed, both before and after extraction, and the difference between the 2 weights taken as the amount of resin extracted. Each sample was composed of spots from a large number of plants and the percentage of resin based on the dry weight of the sample before extraction.

The brown spots from seedling and sapling needles yielded 9.33 and 16.46 per cent resin, respectively, while the bar-spots from saplings, *i.e.*, the type of infection in which the fungus had made the least headway, yielded 20.89 per cent.

A second set of samples was extracted with xylol at 80° C. and showed 7.11 and 13.90 per cent resin in brown spots of seedlings and saplings, respectively. Xylol extractions of spots on definitely susceptible and definitely resistant seedlings showed 6.16 per cent resin in the former and 11.60 per cent in the latter.

The same difference in the ability to produce resin can be shown by cutting off the top halves of needles of nearly disease-free seedlings and saplings and extracting the resin that exudes and hardens on the cut surfaces. Five days after cutting, xylol extractions of the cut surfaces ($\frac{1}{8}$-in. sections back from the cut) showed that saplings produce 23.60 and seedlings 13.69 per cent. resin.

In another and later set, resistant and susceptible seedlings exuded 22.84 and 15.71 per cent resin, respectively.

Bar-spot infection is more common on slash pine, *Pinus caribaea*, and on longleaf pine, *P. palustris*, than on shortleaf pine, *P. echinata*, and loblolly

pine, *P. taeda*. The first two are the highest resin producers and are the trees commonly used as American producers of turpentine. The tree employed for this purpose in Europe, *P. pinaster* Ait., seems to produce mostly bar-spots, as far as can be determined from the few samples present near Bogalusa, Louisiana. Thus, bar-spots, in which the fungus makes the least headway, are more common on the hosts that have the greatest ability to produce resin.

## CONCLUSION

All the evidence indicates that one of the factors responsible for the resistance of saplings and certain seedlings of *Pinus palustris* to the brown-spot fungus, *Septoria acicola*, is related to the ability of the host to produce resin and thus limit the extension of the fungus within the host. This inhibiting influence of the resin may be due either to a toxic effect or, more likely, to a change in the moisture conditions, or to the resin's acting as a mechanical barrier.

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# SEED TRANSMISSION OF VERTICILLIUM WILT OF EGGPLANTS AND TOMATOES

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(Accepted for publication March 9, 1934)

Although wilts caused by *Verticillium* spp. have been studied by a great many workers and have been reported from numerous host plants (3), so far as the writer is aware there are in the literature only 2 references to seeds as possible carriers. Wollenweber (5) isolated *Verticillium* sp. from wheat seed in Germany, but did not establish its pathogenicity. Richardson (2) planted seeds of the eggplant that showed vascular discoloration and had 6 plants die of typical Verticillium wilt. He did not eliminate the possibility of infection by soil-borne Verticillium. He also mentions a seed isolation but gives no details of the technic followed. Since proof of seed transmission of Verticillium wilt is not altogether conclusive, publication of the following investigation seems timely.

Verticillium wilt of eggplant usually is attributed to *Verticillium albo-atrum* R. & B. (4), but Richardson (2) regards the causal fungus as *Verticillium Dahliae* Kleb. He bases his conclusion upon the presence of pseudosclerotia in culture. Verticillium-wilt fungi, with and without pseudosclerotia, have been isolated from eggplants by the writer, although in the great majority of cases they were present.

To determine whether or not the wilt fungus is carried by the seeds of eggplant and tomato, the problem was divided into external and internal carriage. To study external carriage, seeds were aseptically removed from eggplants and tomatoes that showed definite vascular discoloration in the fruits. Some were plated in Petri dishes on 2 per cent potato-dextrose agar immediately upon removal, while others were placed in sterile flasks and allowed to dry at room temperature for 2 months before plating. The results are given in table 1.

TABLE 1.—*Verticillium infestation of the seeds of eggplant and tomato*

| Number of seeds | Kind of seeds | Time of plating | Results[a] |
|---|---|---|---|
| 100 | Eggplant | At removal from berry. | 54 + |
| 100 | Eggplant | Two months after removal | 39 + |
| 25 | Tomato | At removal from berry | 10 + |
| 25 | Tomato | Two months after removal | 14 + |

[a] Plus (+) indicates that the fungus was identified as being culturally identical to that isolated from stems of wilted eggplants and tomatoes.

A portion of the eggplant seeds that had been aseptically removed to sterile flasks and then allowed to dry for 2 months was shaken for 15 minutes in sterile water. A poured-plate series of potato-dextrose agar containing 2 cc. of the wash water was made. After 6 days an examination of the 25 poured plates revealed 7 typical colonies of Verticillium. About 200 seeds were involved in this test.

Possible internal seed infection seemed the most important aspect of the problem, since, in its presence, seed-sterilization methods would necessarily

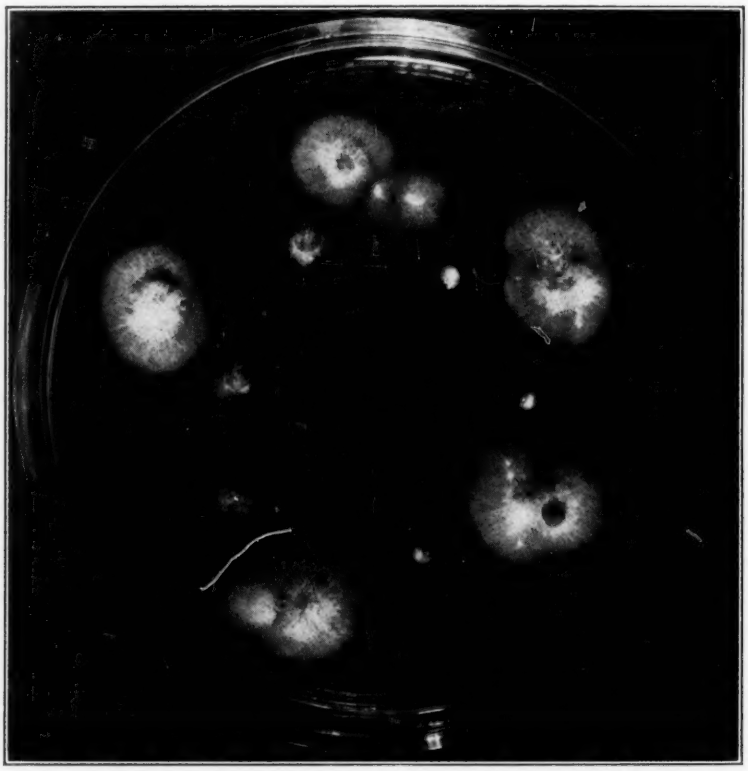

FIG. 1. Verticillium isolated from interior of eggplant seed.

be limited. Seeds for this study were secured from fruits with discolored bundles, but were separated from the fleshy part of the fruit by the usual fermentation method. One group of seeds used in the test were taken at random from commercial packets. The method of sterilization was principally the same as that used by the writer and L. K. Jones (1) when studying seed transmission of Fusarium wilt of peas. In brief, 1 gram of calcium hypochlorite is used to every 14 cc. of sterile distilled water. The

mixture is stirred frequently and after 15 minutes is filtered. The seeds to be treated are allowed to stand with frequent stirring for 1½ to 2 hours in about 5 times their volume of the filtrate. (Since the amount of available chlorine varies considerably with different lots of calcium hypochlorite, the exact time for sterilization without injuring the seeds to be treated must be determined by actual trial.) After treatment they were removed to 2 per cent potato-dextrose agar and allowed sufficient time to develop. To establish the toxicity of this treatment to the wilt fungus, a culture containing pseudosclerotia was placed in a portion of the filtrate, and, after intervals of one-half hour, parts of it were washed thoroughly in sterile distilled water and plated on 2 per cent potato-dextrose agar. All treatments including the ½-hr. treatment were toxic. Similar bits of the fungus without treatment, but washed in distilled water and plated, grew readily. Figure 1 shows a plate of eggplant seeds treated as above with an unusually high percentage of seed infection.

The pathogenicity of typical cultures was established by inoculating sterilized soil with plate cultures and planting it to eggplant from seeds especially selected from wilt-free plants. Checks were grown on sterilized soil from the seeds similarly selected. The checks remained healthy, while those in the inoculated soil showed the typical wilt. Reisolations showed the fungus to be culturally identical to that used for inoculation. Table 2 gives the results of the internal seed carriage tests.

TABLE 2.—*Verticillium-infection of seeds of eggplant and tomato*

| Number of seeds | Kind of seeds | Sources of seeds | Results |
|---|---|---|---|
| 100 | Eggplant | Diseased fruit | 31+ |
| 100 | " | " " | 24+ |
| 200 | " | Commercial packets | 3+ |
| 25 | Tomato | Diseased fruit | 11+ |

It is to be remembered that all the seeds, except those of one commercial lot, were specially selected and, therefore, probably represent a much greater percentage of infected seed than would generally be encountered. It does not necessarily follow, however, that certain lots of commercial seeds could not be as badly infected. Many cases have been observed in Illinois where the eggplant appeared perfectly healthy, but an examination of the fruits thereon showed some vascular discoloration. Isolations from such fruits gave typical Verticillium cultures.

In limited tests of eggplant seeds the hot-water treatment at 120° F. for 20 minutes seemed to control the disease. Because of a shortage of seeds, hot-water tests were not run with tomatoes, and, for the same reason,

only a limited number of tests were made of eggplants. A test-tube culture of the fungus containing pseudosclerotia was not killed by a similar treatment. The pseudosclerotia, however, seemed to be the only portion of the culture to survive.

Seed separation by the usual fermentation method did not seem materially to reduce the amount of seed infection in either eggplants or tomatoes. Selection of clean seed is highly recommended.

## SUMMARY AND CONCLUSIONS

1. The Verticillium-wilt fungus may be carried by tomato and eggplant seeds. Internal carriage seems to be the most important method and may account for the apparent coexistence of the disease with eggplant culture in Illinois. At the present time, the extent of Verticillium wilt of tomatoes in this State is not known.

2. A hot-water treatment at 120° F. for 20 minutes may satisfactorily sterilize infected seeds of eggplant, but this point is not definitely established. Selection of disease free seed is highly recommended.

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# CHEMICAL STUDIES ON THE VIRUS OF TOBACCO MOSAIC
## II. THE PROTEOLYTIC ACTION OF PEPSIN

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(Accepted for publication Sept. 26, 1934)[1]

### INTRODUCTION

Experiments with tobacco-mosaic virus and trypsin already described (8) showed that certain host plants were not susceptible to virus in the presence of trypsin, and that this was due chiefly to an effect of enzyme on the test plant and not, as had been thought previously (6), to proteolytic action of enzyme on virus. Consequently, no evidence as to whether or not the virus is a protein was obtained. However, if the virus of tobacco mosaic is a protein, it seems likely that enzymatic hydrolysis might occur on exposure to some other proteolytic enzyme. The demonstration of such proteolytic hydrolysis, and more particularly of a rate of hydrolysis, would be of interest since it would indicate that the virus is a protein or very closely associated with a protein. The enzyme pepsin was selected for study in this connection because its proteolytic activity extends over a very wide range of substrates and because it was available in a very pure form. Previous workers have, of necessity, used pepsin in the presence of other enzymes, since, until recently, it has been available only in mixtures. Flexner, Clark, and Dochez (2) studied the effect of placing the virus of poliomyelitis in the stomach of monkeys, thus subjecting it to the action of pepsin and other enzymes, and found that such treatment did not inactivate the virus. Lojkin and Vinson (6) reported that pepsin had no action on tobacco-mosaic virus, and Caldwell (1) found that pepsin had no effect on aucuba-mosaic virus. The present paper records the results obtained in a study of the proteolytic action of pepsin on the virus of tobacco mosaic.

### MATERIALS AND METHODS

The virus used in the experiments to be reported consisted of untreated infectious juice and a purified virus preparation. The untreated infectious juice was obtained by pressing plants of mosaic-diseased *Nicotiana tabacum* L. variety Turkish that had been grown in a greenhouse in winter. The plants were cut and kept in a freezing room for a few days and then allowed to thaw just before pressing. The juice was filtered through two layers of bandage gauze and then diluted with two volumes of distilled water. The purified virus was prepared from infectious juice by pre-

[1] Published at the expense of The Rockefeller Institute for Medical Research out of the order determined by the date of acceptance of the manuscript.

cipitation with lead acetate and elution with 0.1 M potassium phosphate at pH 7. The procedure described by Vinson and Petre (9) was modified somewhat for the preparation of purified virus used in this work. The purified virus was diluted with 9 parts of 0.1 M potassium phosphate at pH 7, so that its infectivity would be comparable to that of the diluted untreated infectious juice described above.

Commercially available Parke-Davis pepsin U. S. P. 1:10,000, and a specially prepared crystalline pepsin[2] were used. Commercial pepsin, which is a mixture of pepsin with small amounts of other enzymes (7), was used in the form of a dry powder, added directly to the virus preparations. The crystalline pepsin, which is believed to be only one enzyme (7), was used in the form of a solution in 0.02 N HCl containing 10 mg. of pepsin nitrogen per cc.

Portions of the virus preparations were taken to pH 3, 4, 5, 6, 7, and 8 by the addition, with vigorous stirring, of 2 N HCl or NaOH. Pepsin was added to portions of each of the above solutions. Due to the fact that the virus preparations were well buffered, the addition of pepsin caused but little change in the hydrogen-ion concentration of the solutions. This change was never over 0.3 pH unit and usually less than 0.1 pH unit. After the addition of pepsin, the solutions were immediately tested for virus, using *Nicotiana glutinosa* L. and in certain instances *Phaseolus vulgaris* L., and then incubated at 37° C. for 97 days. The solutions were tested for virus infectivity 6 times during the incubation period in order to follow the course of the reaction. These tests were made by the half-leaf method, as previously described (8). Each of the virus-plus-pepsin preparations was tested against a control that consisted of another portion of the same virus preparation to which no pepsin had been added. In every instance, therefore, the control-virus preparation was exactly the same as the corresponding pepsin-virus preparation, except for the absence of pepsin. *N. glutinosa* was used as the test plant in the majority of the experiments, since its response to virus was found to vary but little over the range pH 3 to pH 8. In addition, *P. vulgaris* was used to test all solutions at pH 4 and pH 7.

## EXPERIMENTAL

A 260 cc. portion of diluted purified virus was taken to pH 3 by the addition of 2 N HCl, with vigorous stirring, and a few drops of toluene were added. To 60 cc. of this preparation was added 60 mg. of commercial pepsin. The mixture was then divided into 3 equal portions. One 20 cc. portion was immediately frozen in 6 small test tubes and allowed to remain in a room held at –15° C. A control solution in 6 small test tubes also was

[2] The writer is indebted to Dr. Northrop and Dr. Herriott for this crystalline pepsin.

placed in this room. The remaining 2 portions were immediately tested for virus infectivity on *Nicotiana glutinosa* against the proper controls and then placed in a room at 37° C. To 2 other 20 cc. portions of the diluted purified virus at pH 3 were added 2 mg. and 200 mg. of commercial pepsin, respectively. These preparations were immediately tested for virus infectivity on *N. glutinosa* against the proper controls, and then placed in a room at 37° C. To other 20 cc. portions of diluted purified virus at pH 4, 5, 6, 7, and 8 were added 20 mg. of commercial pepsin, respectively. These preparations were likewise immediately tested for virus infectivity on *N. glutinosa* against controls at the different hydrogen-ion concentrations. The preparations at pH 4 and pH 7 were also tested on *Phaseolus vulgaris*. These preparations and controls for all the preparations described above were placed in a room at 37° C.

The above described procedure was repeated, using diluted untreated infectious juice in place of the diluted purified virus preparation. Then the procedures just described, involving the use of commercial pepsin with diluted purified virus, and with diluted untreated infectious juice, were repeated, using 0.01 cc. of a crystalline pepsin solution containing 10 mg. of pepsin nitrogen per cc. in place of each milligram of commercial pepsin. It will be seen that a study of the action of commercial pepsin at a concentration of 1 mg. per cc., and of crystalline pepsin at a concentration of 0.1 mg. of pepsin nitrogen per cc., on both diluted purified virus and diluted untreated infectious juice at pH 3, 4, 5, 6, 7, and 8 at 37° C., was included. The effects of increasing the pepsin concentration 10 times, of decreasing it to 1/10, and of carrying out the digestion at –15° C., were studied at pH 3. All preparations, except the frozen samples, were tested for virus infectivity on *Nicotiana glutinosa*, and in certain instances on *Phaseolus vulgaris*, immediately after addition of pepsin, and were then placed in a room at 37° C. After 2 days, portions of all preparations, including the frozen samples, were removed and again tested for virus. This was repeated at the end of 7, 14, 28, and 97 days. In addition, the 4 preparations containing the largest amount of pepsin were tested for virus at the end of 4 days.

A descriptive summary of the 40 different preparations of virus plus pepsin, including the hydrogen-ion and pepsin concentration of each, whether incubated or frozen, and the results of the tests for virus infectivity at the end of the various time periods, is given in tables 1, 2, 3, and 4. The average of the actual number of lesions per half-leaf for each preparation at the end of each period of digestion is given in the rows labeled "Actual." A number, which represents the quotient of the average number of lesions per half-leaf obtained with the virus-plus-pepsin preparation, divided by the average number of lesions per half-leaf obtained on the other halves of the same leaves with the control preparation, multiplied by 100,

TABLE 1.—*The action of commercial pepsin on diluted purified tobacco-mosaic virus*

| Temperature of incubation | pH of solution | Mg. pepsin per cc. | Test plant | Tested → | Immediately | After 2 days | After 4 days | After 7 days | After 14 days | After 28 days | After 97 days |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 37° C. | 3 | 10.0 | N. glutinosa | Average[a] | 94.7 | 17.7 | 1.7 | 0.0 | 0.0 | 0.0 | 0.0 |
| | | | | Actual[b] | 56.5 | 7.2 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 |
| 37° C. | 3 | 0.1 | " | Average | 142.0 | 54.4 | | 27.4 | 16.1 | 22.2 | 0.0 |
| | | | | Actual | 19.8 | 35.7 | | 13.6 | 1.5 | 1.6 | 0.0 |
| 37° C. | 3 | 1.0 | " | Average | 156.0 | 87.1 | | 24.6 | 9.3 | 0.0 | 0.0 |
| | | | | Actual | 37.5 | 14.9 | | 2.1 | 0.4 | 0.0 | 0.0 |
| 37° C. | 3 | 1.0 | " | Average | 64.3 | 56.3 | | 19.8 | 1.7 | 0.0 | 0.0 |
| | | | | Actual | 26.6 | 19.6 | | 5.0 | 0.1 | 0.0 | 0.0 |
| –15° C. | 3 | 1.0 | " | Average | | 94.0 | | 103.7 | 209.0 | 94.0 | 110.0 |
| | | | | Actual | | 37.4 | | 14.3 | 7.1 | 4.7 | 1.1 |
| 37° C. | 4 | 1.0 | " | Average | 143.0 | 148.0 | | 81.6 | 55.0 | 44.2 | 23.0 |
| | | | | Actual | 11.7 | 16.2 | | 24.4 | 9.5 | 3.8 | 3.9 |
| 37° C. | 4 | 1.0 | P. vulgaris | Average | 18.2 | 32.4 | | 7.2 | 23.6 | 3.4 | 0.0 |
| | | | | Actual | 8.3 | 19.0 | | 3.1 | 21.4 | 1.5 | 0.0 |
| 37° C. | 5 | 1.0 | N. glutinosa | Average | 100.0 | 86.5 | | 99.4 | 109.5 | 90.6 | 133.0 |
| | | | | Actual | 15.2 | 15.2 | | 29.2 | 20.4 | 9.7 | 10.8 |
| 37° C. | 6 | 1.0 | " | Average | 97.2 | 108.0 | | 116.0 | 106.1 | 75.0 | 134.0 |
| | | | | Actual | 26.9 | 21.2 | | 49.8 | 23.7 | 7.5 | 24.5 |
| 37° C. | 7 | 1.0 | " | Average | 132.0 | 72.0 | | 100.0 | 77.0 | 102.0 | 139.0 |
| | | | | Actual | 44.8 | 19.4 | | 27.5 | 33.0 | 42.6 | 51.0 |
| 37° C. | 7 | 1.0 | P. vulgaris | Average | 41.6 | 42.0 | | 46.7 | 48.7 | 21.8 | 12.0 |
| | | | | Actual | 85.4 | 56.2 | | 89.0 | 83.6 | 22.1 | 8.7 |
| 37° C. | 8 | 1.0 | N. glutinosa | Average | 114.0 | 89.0 | | 98.8 | 70.0 | 155.0 | 110.2 |
| | | | | Actual | 36.0 | 18.5 | | 7.7 | 6.0 | 8.2 | 8.4 |

[a] Numbers represent the average number of lesions per half-leaf obtained on 7 or more half-leaves of *Nicotiana glutinosa* or 16 or more half-leaves of *Phaseolus vulgaris* on inoculation with the designated virus-plus-pepsin preparation divided by the average number of lesions per half-leaf obtained on the other halves of the same leaves with the corresponding control preparation, multiplied by 100.

[b] Numbers represent the average of the actual number of lesions per half-leaf obtained on 7 or more half-leaves of *N. glutinosa* or 16 or more half-leaves of *P. vulgaris* on inoculation with the designated virus-plus-pepsin preparation.

TABLE 2.—*The action of commercial pepsin on diluted untreated infectious juice*

TABLE 2.—*The action of commercial pepsin on diluted untreated infectious juice*

| Temperature of incubation | pH of solution | Mg. pepsin per cc. | Test plant | Tested → | Immediately | After 2 days | After 4 days | After 7 days | After 14 days | After 28 days | After 97 days |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 37° C. | 3 | 10.0 | N. glutinosa | Average[a] | 69.4 | 8.7 | 4.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | | | | Actual[b] | 70.7 | 10.0 | 0.9 | 0.0 | 0.0 | 0.0 | 0.0 |
| 37° C. | 3 | 0.1 | " | Average | 130.0 | 110.0 | | 35.1 | 17.7 | 35.0 | 16.0 |
| | | | | Actual | 14.5 | 23.0 | | 23.9 | 6.0 | 2.6 | 1.0 |
| 37° C. | 3 | 1.0 | " | Average | 143.0 | 113.0 | | 44.2 | 3.2 | 2.2 | 1.7 |
| | | | | Actual | 33.4 | 32.8 | | 15.4 | 0.5 | 0.4 | 0.3 |
| 37° C. | 3 | 1.0 | " | Average | 112.0 | 68.5 | | 30.6 | 0.0 | 0.0 | 0.0 |
| | | | | Actual | 55.8 | 24.4 | | 17.9 | 0.0 | 0.0 | 0.0 |
| –15° C. | 3 | 1.0 | " | Average | | 101.0 | | 124.7 | 208.0 | 163.0 | 147.0 |
| | | | | Actual | | 26.9 | | 43.7 | 16.2 | 23.2 | 12.2 |
| 37° C. | 4 | 1.0 | " | Average | 135.0 | 94.0 | | 93.0 | 70.5 | 61.0 | 59.0 |
| | | | | Actual | 22.6 | 62.5 | | 31.4 | 15.3 | 12.4 | 16.6 |
| 37° C. | 4 | 1.0 | P. vulgaris | Average | 26.6 | 45.2 | | 36.4 | 16.0 | 11.5 | 11.0 |
| | | | | Actual | 3.6 | 14.3 | | 4.1 | 6.5 | 3.6 | 1.0 |
| 37° C. | 5 | 1.0 | N. glutinosa | Average | 110.0 | 79.4 | | 74.3 | 45.0 | 84.0 | 61.0 |
| | | | | Actual | 39.6 | 27.6 | | 20.8 | 10.7 | 16.5 | 19.9 |
| 37° C. | 6 | 1.0 | " | Average | 98.6 | 66.1 | | 81.9 | 78.2 | 91.0 | 65.2 |
| | | | | Actual | 45.2 | 27.0 | | 34.0 | 24.0 | 20.3 | 35.5 |
| 37° C. | 7 | 1.0 | " | Average | 101.5 | 112.0 | | 109.5 | 97.5 | 121.0 | |
| | | | | Actual | 28.3 | 20.9 | | 72.8 | 25.9 | 33.2 | |
| 37° C. | 7 | 1.0 | P. vulgaris | Average | 34.4 | 32.2 | | 78.6 | 56.9 | 38.0 | |
| | | | | Actual | 7.2 | 16.6 | | 18.4 | 49.2 | 11.7 | |
| 37° C. | 8 | 1.0 | N. glutinosa | Average | 97.5 | 80.5 | | 105.5 | 123.0 | 103.8 | 104.0 |
| | | | | Actual | 43.7 | 84.5 | | 40.8 | 18.1 | 16.6 | 37.8 |

[a] Numbers represent the average number of lesions per half-leaf obtained on 7 or more half-leaves of *Nicotiana glutinosa* or 16 or more half-leaves of *Phaseolus vulgaris* on inoculation with the designated virus-plus-pepsin preparation divided by the average number of lesions per half-leaf obtained on the other halves of the same leaves with the corresponding control preparation, multiplied by 100.

[b] Numbers represent the average of the actual number of lesions per half-leaf obtained on 7 or more half-leaves of *N. glutinosa* or 16 or more half-leaves of *P. vulgaris* on inoculation with the designated virus-plus-pepsin preparation.

is also given for each preparation. This number, which is given in the rows labeled "Average," may be considered a measure of the virus present. It is recognized that the number may not be strictly proportional to the actual amount of virus. However, it serves as an indication of the amount of virus present and enables one to determine rapidly whether much, little, or no virus is left in the pepsin-treated solutions at the end of the various digestion periods. As long as this number remains near 100, it indicates that the pepsin-treated virus preparation produces about as many lesions as does the corresponding control preparation; hence much of the original virus must be present. As this number decreases, it indicates that a decreasing amount of virus is present in the pepsin-treated virus preparation. When no lesions are produced on inoculation of *Nicotiana glutinosa* or *Phaseolus vulgaris* with the pepsin-treated virus preparation, the number becomes zero.

The results given in columns headed by "At once" in tables 1 to 4 show that neither commercial nor crystalline pepsin has much immediate effect on the infectivity of either purified tobacco-mosaic virus or infectious juice over the range of pH 3 to pH 8, as measured on *Nicotiana glutinosa*. *Phaseolus vulgaris* was found to give erratic results, especially at pH 4. Because of previous work, it had been expected that the preparations at pH 4 or lower might give irregular results on *P. vulgaris*, but it was hoped that satisfactory results might be obtained at pH 7. It is obvious, however, that *P. vulgaris* is not a suitable test plant for the present experiments, since, even at pH 7, the susceptibility of the plants to virus is changed when pepsin is present. Consequently, in the following paragraphs, only the results obtained with *N. glutinosa* will be considered.

It may be seen further from tables 1, 2, 3, and 4 that neither 1 mg. commercial pepsin per cc. nor 0.1 mg. crystalline pepsin nitrogen per cc. has any appreciable effect on the infectivity of either purified tobacco-mosaic virus or diluted untreated infectious juice when incubated at 37° C. for several days at either pH 7 or pH 8. These pepsin-treated virus samples produced about as many lesions on inoculation as did the corresponding controls. When the hydrogen-ion concentration of the digestion mixtures was lowered to pH 5 or pH 6, there was a slight tendency for the infectivity of the virus-pepsin preparations to drop on prolonged digestion. This tendency became quite marked when the hydrogen-ion concentration of the digestion mixtures was further lowered to pH 4. At this hydrogen-ion concentration the pepsin-treated virus preparations produced only 2.1, 32.2, 23.0, and 59.0 per cent as many lesions on *Nicotiana glutinosa* after 97 days' incubation as did the corresponding controls. Furthermore, there was a definite trend to the reduction in infectivity, the pepsin-treated preparations, in general, producing a smaller percentage of lesions after each suc-

TABLE 3.—*The action of crystalline pepsin on diluted purified*

TABLE 3.—*The action of crystalline pepsin on diluted purified tobacco-mosaic virus*

| Temperature of incubation | pH of solution | Mg. pepsin nitrogen per cc. | Test plant | Tested → | Immediately | After 2 days | After 4 days | After 7 days | After 14 days | After 28 days | After 97 days |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 37° C. | 3 | 1.0 | N. glutinosa | Average[a] | 62.6 | 11.8 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 |
| | | | | Actual[b] | 24.5 | 6.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| 37° C. | 3 | 0.01 | " | Average | 93.5 | 53.4 | | 32.8 | 17.8 | 2.5 | 2.7 |
| | | | | Actual | 15.9 | 32.4 | | 17.2 | 0.8 | 0.4 | 0.3 |
| 37° C. | 3 | 0.1 | " | Average | 111.8 | 60.5 | | 16.0 | 0.0 | 0.0 | 0.0 |
| | | | | Actual | 24.2 | 28.1 | | 4.1 | 0.0 | 0.0 | 0.0 |
| 37° C. | 3 | 0.1 | " | Average | 88.4 | 46.0 | | 9.1 | 0.0 | 0.0 | 0.0 |
| | | | | Actual | 52.0 | 15.4 | | 2.5 | 0.0 | 0.0 | 0.0 |
| −15° C. | 3 | 0.1 | " | Average | | 73.5 | | 91.0 | 170.0 | 93.0 | 84.5 |
| | | | | Actual | | 12.1 | | 13.0 | 4.6 | 4.0 | 1.6 |
| 37° C. | 4 | 0.1 | " | Average | 107.8 | 108.5 | | 69.4 | 57.9 | 27.7 | 2.1 |
| | | | | Actual | 37.4 | 66.7 | | 31.8 | 11.7 | 3.6 | 0.3 |
| 37° C. | 4 | 0.1 | P. vulgaris | Average | 48.9 | 24.6 | | 29.8 | 48.3 | 9.5 | 0.1 |
| | | | | Actual | 18.5 | 19.6 | | 15.2 | 76.4 | 3.0 | 0.1 |
| 37° C. | 5 | 0.1 | N. glutinosa | Average | 122.0 | 81.2 | | 72.8 | 59.0 | 45.7 | 48.0 |
| | | | | Actual | 29.8 | 22.0 | | 14.7 | 15.7 | 12.2 | 6.9 |
| 37° C. | 6 | 0.1 | " | Average | 85.2 | 129.0 | | 85.0 | 65.0 | 95.5 | 88.0 |
| | | | | Actual | 35.0 | 42.5 | | 37.8 | 20.6 | 28.3 | 34.2 |
| 37° C. | 7 | 0.1 | " | Average | 68.4 | 102.5 | | 92.8 | 103.8 | 78.3 | 88.2 |
| | | | | Actual | 30.4 | 21.3 | | 29.7 | 25.1 | 31.5 | 34.2 |
| 37° C. | 7 | 0.1 | P. vulgaris | Average | 56.5 | 101.0 | | 107.1 | 78.6 | 62.0 | 59.5 |
| | | | | Actual | 101.0 | 149.0 | | 134.4 | 146.1 | 108.5 | 64.2 |
| 37° C. | 8 | 0.1 | N. glutinosa | Average | 57.0 | 76.5 | | 68.8 | 130.0 | 101.5 | 81.0 |
| | | | | Actual | 17.0 | 8.8 | | 9.0 | 10.0 | 6.7 | 8.4 |

[a] Numbers represent the average number of lesions per half-leaf obtained on 7 or more half-leaves of *Nicotiana glutinosa* or 16 or more half-leaves of *Phaseolus vulgaris* on inoculation with the designated virus-plus-pepsin preparation divided by the average number of lesions per half-leaf obtained on the other halves of the same leaves with the corresponding control preparation, multiplied by 100.

[b] Numbers represent the average of the actual number of lesions per half-leaf obtained on 7 or more half-leaves of *N. glutinosa* or 16 or more half-leaves of *P. vulgaris* on inoculation with the designated virus-plus-pepsin preparation.

TABLE 4.—*The action of crystalline pepsin on diluted untreated infectious juice*

| Temperature of incubation | pH of solution | Mg. pepsin nitrogen per cc. | Test plant | Tested → | Immediately | After 2 days | After 4 days | After 7 days | After 14 days | After 28 days | After 97 days |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 37° C. | 3 | 1.0 | N. glutinosa | Average[a] | 77.3 | 15.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | | | | Actual[b] | 37.6 | 14.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 37° C. | 3 | 0.01 | " | Average | 83.0 | 80.2 | | 40.8 | 28.7 | 25.5 | 4.4 |
| | | | | Actual | 12.2 | 64.0 | | 32.6 | 5.4 | 7.2 | 0.8 |
| 37° C. | 3 | 0.1 | " | Average | 111.0 | 68.3 | | 24.8 | 0.0 | 0.0 | 0.0 |
| | | | | Actual | 64.3 | 25.3 | | 14.4 | 0.0 | 0.0 | 0.0 |
| 37° C. | 3 | 0.1 | " | Average | 102.3 | 70.5 | | 22.7 | 0.0 | 0.0 | 0.0 |
| | | | | Actual | 74.3 | 27.0 | | 13.9 | 0.0 | 0.0 | 0.0 |
| –15° C. | 3 | 0.1 | " | Average | | 114.0 | | 89.4 | 113.6 | 80.6 | 139.0 |
| | | | | Actual | | 34.6 | | 38.4 | 23.2 | 24.2 | 22.4 |
| 37° C. | 4 | 0.1 | " | Average | 92.5 | 74.5 | | 70.0 | 63.1 | 67.8 | 32.2 |
| | | | | Actual | 38.6 | 53.3 | | 42.0 | 37.6 | 27.0 | 10.5 |
| 37° C. | 4 | 0.1 | P. vulgaris | Average | 33.8 | 43.0 | | 24.4 | 41.5 | 54.5 | 18.0 |
| | | | | Actual | 5.1 | 22.9 | | 6.2 | 27.2 | 11.3 | 2.2 |
| 37° C. | 5 | 0.1 | N. glutinosa | Average | 90.5 | 57.0 | | 69.5 | 116.6 | 99.3 | 66.0 |
| | | | | Actual | 26.4 | 49.2 | | 53.1 | 38.3 | 24.4 | 26.9 |
| 37° C. | 6 | 0.1 | " | Average | 55.5 | 116.0 | | 55.5 | 83.2 | 67.3 | 90.0 |
| | | | | Actual | 27.3 | 17.8 | | 29.8 | 36.6 | 18.5 | 54.8 |
| 37° C. | 7 | 0.1 | " | Average | 79.8 | 123,0 | | 108.0 | 99.0 | 92.5 | 128.0 |
| | | | | Actual | 41.5 | 34.4 | | 69.3 | 29.7 | 32.2 | 46.9 |
| 37° C. | 7 | 0.1 | P. vulgaris | Average | 45.8 | 75.5 | | 53.0 | 64.0 | 31.8 | 58.9 |
| | | | | Actual | 20.0 | 36.0 | | 30.2 | 65.9 | 24.0 | 50.7 |
| 37° C. | 8 | 0.1 | N. glutinosa | Average | 67.6 | 53.5 | | 105.0 | 100.0 | 94.7 | 121.0 |
| | | | | Actual | 34.7 | 61.5 | | 30.5 | 32.6 | 15.7 | 30.6 |

[a] Numbers represent the average number of lesions per half-leaf obtained on 7 or more half-leaves of *Nicotiana glutinosa* or 16 or more half-leaves of *Phaseolus vulgaris* on inoculation with the designated virus-plus-pepsin preparation divided by the average number of lesions per half-leaf obtained on the other halves of the same leaves with the corresponding control preparation, multiplied by 100.

[b] Numbers represent the average of the actual number of lesions per half-leaf obtained on 7 or more half-leaves of *N. glutinosa* or 16 or more half-leaves of *P. vulgaris* on inoculation with the designated virus-plus-pepsin preparation.

ceeding period of digestion. When the hydrogen-ion concentration of the digestion mixtures was dropped to pH 3, the preparations containing 0.1 mg. of crystalline pepsin nitrogen per cc., with either purified virus or diluted infectious juice, produced no lesions on inoculation to *N. glutinosa* after 14 days' incubation at 37° C. The preparations of purified virus with 1 mg. of commercial pepsin per cc. required more than 14 days' incubation before loss of virus infectivity occurred. One preparation of diluted untreated juice plus commercial pepsin failed to produce lesions on inoculation to *N. glutinosa* after 14 days' incubation, while the other similar preparation still gave a few lesions after 28 days' incubation. When the amount of either crystalline or commercial pepsin was increased 10 times, the length of incubation necessary for a preparation that produced no lesions on inoculation to *N. glutinosa* was shortened markedly, in one case to less than 4 days, and in 3 other cases to less than 7 days. When the amount of either of the pepsin preparations was decreased to 1/10, 3 out of the 4 preparations produced a few lesions on inoculation to *N. glutinosa*, even after 97 days' incubation at 37° C. Furthermore, it may be seen that when the temperature of incubation was dropped from 37° C. to –15° C., all of the pepsin-treated virus preparations produced about as many lesions on inoculation to *N. glutinosa* as did the corresponding control preparations, even after a digestion period of 97 days.

A graphic representation of the results is given in figures 1 and 2. The results obtained with commercial pepsin and crystalline pepsin with both purified virus and infectious juice at the end of the various periods of digestion were averaged for each hydrogen-ion concentration and for each pepsin concentration and then plotted on a logarithmic scale against the time of digestion in days. In figure 1, the average of the number of lesions obtained with the 8 digestion mixtures listed in tables 1 to 4 containing 1.0 mg. commercial pepsin or 0.1 mg. crystalline pepsin nitrogen per cc. at pH 3 and 37° C., expressed as a percentage of the average number of lesions obtained with the controls, is plotted against the time of digestion. The results obtained with the preparations, of which there were 4 each at pH 4, 5, 6, 7 and 8, and containing comparable amounts of pepsin, were similarly treated and are also given in figure 1. It may be seen that the lines showing the results obtained at pH 5, 6, 7 and 8 fall very close to the line for the controls. This indicates that in each case the digestion mixtures produced about as many lesions as the corresponding control at the end of each period of digestion. The line for the results obtained at pH 4 gradually falls away from the control line, indicating a decreasing number of lesions with time of digestion. The line for the results obtained at pH 3 falls away sharply, thus indicating a sharp decrease in the average number of lesions at this hydrogen-ion concentration. Figure 2 pictures the results

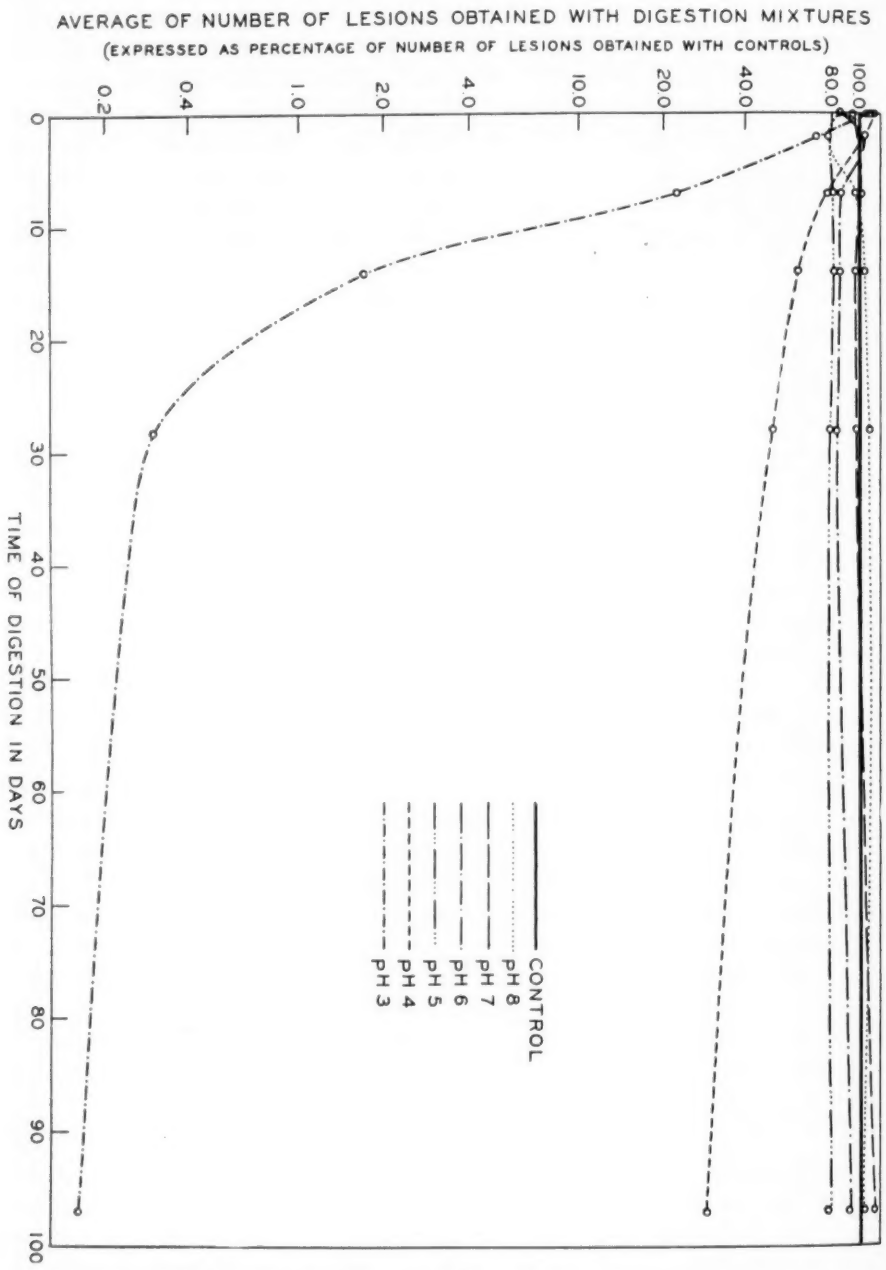


FIG. 1. Graph showing the effect of different hydrogen-ion concentrations on the inactivation of tobacco-mosaic virus with pepsin at 37° C. The lines showing the average results obtained at pH 5, 6, 7 and 8 fall very close to the line for the controls, thus indicating little or no inactivation of virus. The line showing the results obtained at pH 3 drops sharply, thus indicating a rapid inactivation of virus.

obtained at pH 3 with 3 different pepsin concentrations and the result when the digestion mixture is maintained at –15° C. instead of 37° C. The line indicating the results obtained with the mixtures containing the greatest pepsin concentration drops off almost at right angles to the control line, whereas the line depicting the results when only 1/100 as much pepsin is present falls off gradually. It also may be seen from figure 2 that the results obtained with an intermediate pepsin concentration at 37° C. give a line that falls off sharply, whereas the results obtained with portions of the same preparations, maintained at –15° C., give a line that falls very close to that of the control.

The results show that pepsin has no appreciable effect on the infectivity of tobacco-mosaic virus at pH 7 or pH 8, and but slight effect at pH 5 or pH 6, even after prolonged incubation at 37° C. However, at pH 4, pepsin has a noticeable effect in reducing the infectivity of virus after prolonged incubation, and at pH 3, pepsin reduces the infectivity of the virus quite rapidly at 37° C. Small amounts of pepsin cause the reduction in infectivity to proceed slowly and, conversely, large amounts of pepsin cause the reaction to proceed rapidly. Amounts of pepsin, which at pH 3 and 37° C. would cause a complete loss of infectivity in a given period, fail to affect the infectivity when the preparations are kept the same length of time at –15° C. Crystalline pepsin appears to be slightly more effective than commercial pepsin. This is due, probably, to the fact that, although the actual weight of commercial pepsin used in comparable experiments was 1.6 times that of crystalline pepsin, the latter is about 5 times more active (7) proteolytically than commercial pepsin. The virus in the purified preparations appears to be affected slightly more by pepsin than that in the diluted, untreated infectious juice. This may be due to the presence of antienzymes or the great amount of extraneous protein matter in the untreated juice.

Although the averaged numbers given in tables 1 to 4 serve to give an idea of the course of the reaction, a better approximation of the percentage of virus remaining in a given virus-pepsin digestion mixture could be obtained by a comparison of the number of lesions produced by the digestion mixture, with the number of lesions produced by dilutions of the control or untreated virus preparation. For example, if, on inoculation, a given digestion mixture produces about the same number of lesions as does the control virus preparation diluted 1 to 10, it should be safe to assume that about 10 per cent of the virus is left. If the number of lesions produced is about the same as that given by the control diluted 1 to 100, then probably only about one per cent of the virus remains. The estimation of the amount of virus left after various digestion periods is useful in arriving at the rate of the reaction.

Since most of the experiments were made at pH 3, portions of the original control preparations at pH 3 used in the tests listed in tables 1 to 4 were diluted 1 to 10 and 1 to 100 and tested on *Nicotiana glutinosa*. The purified virus was diluted with 0.1 M phosphate at pH 3, and the infectious juice was diluted with 0.001 N HCl in order to give solutions at pH 3. There is some loss of virus on standing at pH 3 and 37° C.; so after standing for 14 days, additional portions of the control preparations were similarly diluted and tested. This makes possible a differentiation between loss of virus due to standing at pH 3 and 37° C., and loss of virus due to the action

TABLE 5.—*Dilution of control preparations of purified virus*[a] *and of infectious juice*[b]

| Dilution | Purified virus | | Infectious juice | |
|---|---|---|---|---|
| | At once | After 14 days at 37° C. | At once | After 14 days at 37° C. |
| 1 | 39.8[c] | 6.8 | 32.2 | 25.2 |
| 1–10 | 8.4 | 1.7 | 5.4 | 4.9 |
| 1–100 | 1.5 | 0.2 | 0.8 | 0.6 |

[a] Dilutions of purified virus were made with 0.1 M phosphate at pH 3.

[b] Dilutions of infectious juice were made with 0.001 N hydrochloric acid (pH 3).

[c] Numbers represent the average number of lesions per half-leaf on 7 or more leaves of *N. glutinosa*.

of pepsin. From the results of the tests, (Table 5) the percentage of virus remaining in the various digestion mixtures at pH 3, listed in tables 1 to 4, may be estimated. It may be seen that digestion mixtures of purified virus, producing about 8 lesions per half-leaf within a few days after starting the digestion, have lost about 90 per cent of the virus because of the action of pepsin, and mixtures giving about 1.5 lesions have lost about 99 per cent of the virus. Similarly, mixtures that have stood for about 14 days and that produce about 1.7 lesions have lost about 90 per cent of the virus, and mixtures that produce about 0.2 lesions have lost about 99 per cent of the virus. In the case infectious juice, digestion mixtures that produce about 5 lesions on inoculation after standing for any period up to 14 days have lost about 90 per cent of the virus, and when they produce about 0.7 lesions about 99 per cent of the virus has disappeared. It is realized that some variation may be expected, because the comparisons described above are between the numbers of lesions produced on different sets of plants inoculated at different times. However, in general, the method is useful.

It may be seen, for example, that 0.1 mg. crystalline pepsin nitrogen or 1 mg. commercial pepsin per cc. with purified virus inactivated about 90 per cent of the virus in 7 days at pH 3 and 37° C. Crystalline pepsin caused 100 per cent inactivation in 14 days, and commercial pepsin about 99 per cent inactivation in 14 days and 100 per cent inactivation in less than 28 days. When the concentration of either form of pepsin was increased 10 times, 90 per cent inactivation occurred in about 2 days, 99 per cent inactivation in about 4 days, and 100 per cent inactivation in less than 7 days. When the concentration of either form of pepsin was decreased 1/10, 90 per cent inactivation of virus did not take place until after 7 days, 99 per cent inactivation until about 28 days, and in the case of commercial pepsin 100 per cent inactivation in less than 97 days. When infectious juice was used at pH 3 with 0.1 mg. crystalline pepsin nitrogen or 1.0 mg. commercial pepsin per cc., it required longer than 14 days to obtain 90 per cent inactivation, and in 3 instances less than 14 days for 100 per cent inactivation. When the pepsin concentration was increased 10 times, less than 90 per cent inactivation occurred in 2 days, and 99 per cent in one case, and 100 per cent in the other case at the end of 4 days. When the pepsin concentration was decreased 1/10 about 90 per cent inactivation occurred after 14 days' digestion and 99 per cent after 97 days' digestion. It may be seen that, in general, the rate of inactivation is directly proportionaly to the amount of pepsin present.

A consideration of the results given above in connection with the characteristics of pepsin (7, 3) as a proteolytic enzyme is desirable. Pepsin is inactive as a proteolytic agent at pH 7 or pH 8. It possesses practically no activity at pH 5 and pH 6, and only about 10 per cent of its maximum activity at pH 4. It has more than 50 per cent of its maximum activity at pH 3 and has its maximum activity at about pH 2. At the concentration used it is practically inactive at low temperatures such as as –15° C., and, like most enzymes, is more active at a higher temperature, such as 37° C. Its activity varies directly with its concentration. Thus, from the results obtained in the experiments described above, and the facts known about the proteolytic activity of pepsin, it becomes obvious that pepsin affects the infectivity of the virus of tobacco mosaic only under the conditions which are favorable for peptic activity. Furthermore, the results indicate that the loss of virus infectivity occurs at a more or less definite rate, inasmuch as the inactivation was found to vary directly with the amount of pepsin present, the activity of the pepsin, and the length of the period of digestion. The results are, therefore, a definite indication that the loss of virus infectivity on peptic digestion is due to the proteolytic action of pepsin. This suggests that the virus of tobacco mosaic is a protein, which is susceptible to hydrolysis in the presence of pepsin.

There remained the possibility, however, that virus is not attacked by pepsin, but that split products of other proteins, present in the digestion mixture, might so affect either the virus, or the plant used to measure its infectivity, as to cause the virus to fail to manifest its infectivity on inoculation. This did not appear very probable, however, since if such were the case, one would expect a greater effect with the untreated infectious juice, which contains much more protein than does the purified virus preparation. As may be seen by a comparison of the results given in table 1 and 3 with those given in tables 2 and 4, such was not the case.

TABLE 6.—*The addition of purified virus to pepsin-inactivated virus preparations*[a]

| | Tested → | At once | After 2 days | After 4 days | After 13 days | After 23 days |
|---|---|---|---|---|---|---|
| 1 volume commercial pepsin—purified virus mixture after 7 days' incubation (listed top table 1) + 1 volume purified virus | Average[b] | 90.1 | 57.7 | 44.5 | 9.0 | 9.0 |
| | Actual[c] | 70.8 | 25.2 | 17.1 | 1.9 | 3.0 |
| 1 volume commercial pepsin—infectious juice mixture after 7 days' incubation (listed top table 2) + 1 volume purified virus | Average | 88.0 | 50.9 | 41.0 | 28.0 | 9.1 |
| | Actual | 111.2 | 34.8 | 13.1 | 4.3 | 4.2 |
| 1 volume crystalline pepsin—purified virus mixture after 7 days' incubation (listed top table 3) + 1 volume purified virus | Average | 94.0 | 73.8 | 43.5 | 19.8 | 12.0 |
| | Actual | 97.4 | 31.6 | 15.4 | 4.1 | 3.2 |
| 1 volume crystalline pepsin—infectious juice mixture after 7 days' incubation (listed top table 4) + 1 volume purified virus | Average | 109.5 | 52.2 | 51.5 | 37.0 | 18.9 |
| | Actual | 80.0 | 34.8 | 26.4 | 9.4 | 9.3 |

[a] All preparations were at pH 3 and incubated at 37° C.

[b] Numbers represent the average number of lesions per half-leaf obtained on 5 or more half-leaves of *Nicotiana glutinosa* on inoculation with the designated virus-pepsin preparation divided by the average number of lesions per half-leaf obtained on the other halves of the same leaves with the corresponding control preparation, multiplied by 100.

[c] Numbers represent the average of the actual number of lesions per half-leaf obtained on 5 or more half-leaves of *N. glutinosa* on inoculation with the designated virus-pepsin preparation.

since the untreated infectious juice was affected less than the purified virus preparation. Furthermore, when purified virus was added to pepsin-virus preparations, which had been subjected to incubation at 37° C. and pH 3 until no lesions appeared on inoculation to *Nicotiana glutinosa*, the infectivity of the added virus was not changed appreciably. The results of such an experiment are given in table 6. Portions of the same 4 pepsin-virus preparations listed at the top of tables 1, 2, 3, and 4, containing 1.0 mg. of crystalline pepsin nitrogen per cc. or 10 mg. of commercial pepsin per cc. with both diluted purified virus and diluted untreated infectious juice, were used after 7 days' incubation at 37° C. and pH 3. One volume of each of the 4 preparations described above was added to an equal volume of a purified virus preparation and the resulting mixtures were immediately tested for virus infectivity by the half-leaf method on *N. glutinosa* against controls. Controls for the 2 pepsin-purified virus preparations consisted of the purified virus preparation plus an equal volume of 0.1 M phosphate at pH 3. Controls for the 2 pepsin-infectious juice preparations consisted of the purified virus preparation plus an equal volume of diluted healthy Turkish tobacco juice at pH 3. The tests for virus infectivity were repeated after incubation at 37° C. for 2, 4, 13, and 23 days. It may be seen from table 6 that the 4 pepsin-treated virus preparations, upon addition of new virus, gave 90.1, 88.0, 94.0, and 109.5 per cent, respectively, as many lesions on *N. glutinosa* as did the corresponding control preparations. The average of the results obtained with the 4 preparations is shown in figure 2 as a continuation of the line giving the results with the highest ($C \times 10$) concentration of pepsin. The results demonstrate that split products in pepsin-virus digestion mixtures have no appreciable effect on either virus added to such mixtures or the plant used to measure the infectivity of the virus. Furthermore, the results given in table 6 and portrayed in figure 2 show that virus, added to a pepsin-virus digestion mixture, is susceptible to digestion, since the infectivity of the added virus decreased on incubation.

Many attempts have been made to regain the infectivity of virus that has disappeared as a result of digestion with pepsin. Heating for 20 minutes at 70° C., or diluting with water, 0.1 M phosphate at pH 7, or healthy Turkish tobacco juice, failed to restore the infectivity of virus inactivated with pepsin, although such procedures increase the infectivity of virus in trypsin-virus mixtures. The addition of infectious juice or purified virus, inactivated by heat or by either high or low hydrogen-ion concentrations, and the addition of activated charcoal to pepsin-inactivated virus failed to restore the infectivity of the virus. Changing the hydrogen-ion concentration of virus preparations inactivated by means of pepsin to pH 5 or pH 7 also failed to cause the infectivity of the virus to return, even

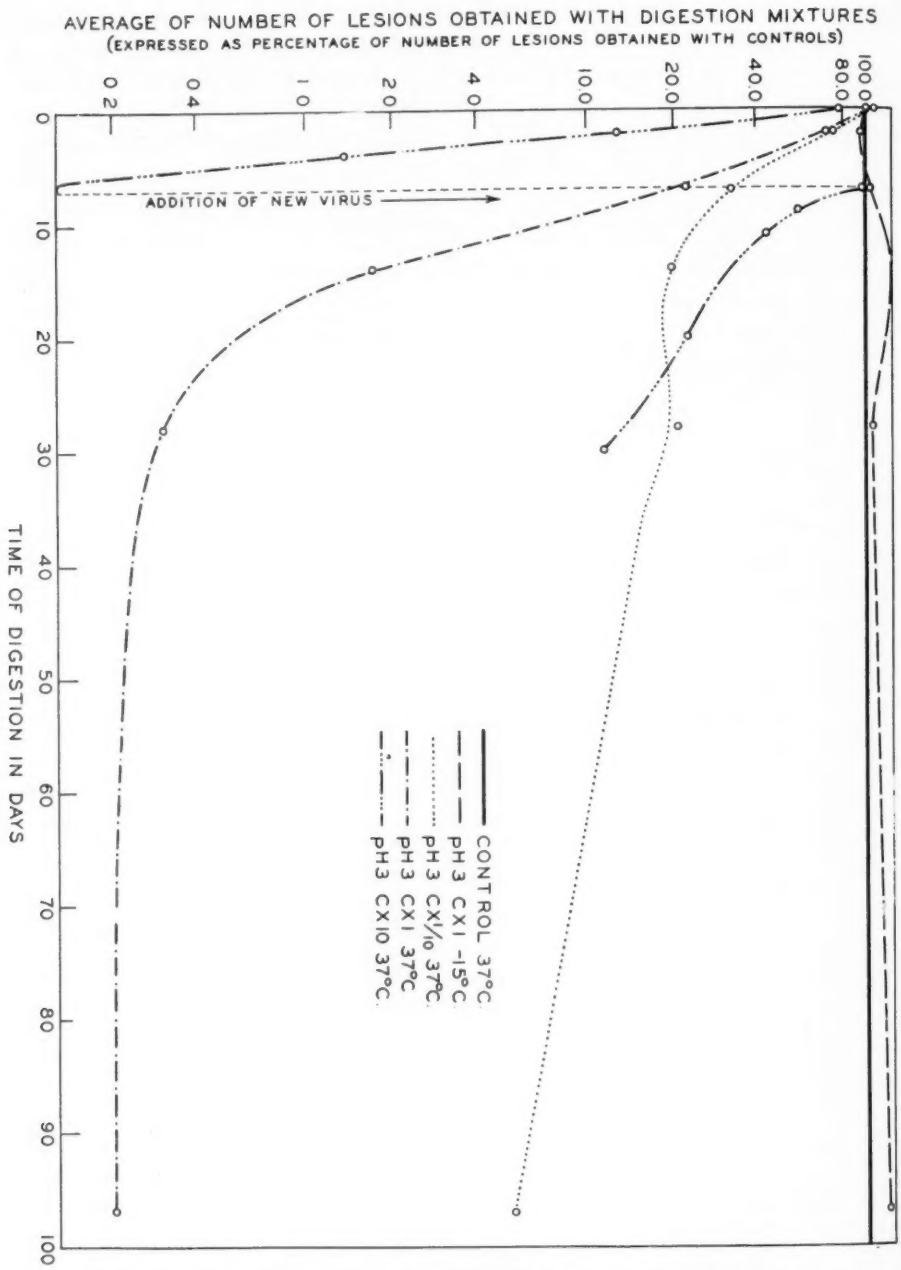


FIG. 2. Graph showing the effect of different pepsin concentrations and of a different digestion temperature on the inactivation of tobacco-mosaic virus with pepsin. The line showing the average results obtained with the pepsin concentration (C×1)

after long standing. It seems difficult to avoid the conclusion that tobacco-mosaic virus is a protein, or closely associated with a protein, which may be hydrolyzed with pepsin.

### The Action of Pepsin on Different Strains of Tobacco-mosaic Virus

An experiment was made for the purpose of determining whether digestion with pepsin would inactive other strains of tobacco-mosaic virus. The strains used in this experiment were aucuba mosaic, a masked strain (4) and a yellow strain (5) of tobacco-mosaic virus previously described (8). The aucuba mosaic was a purified sample prepared from infectious tomato juice. The masked and yellow strains were in juice from infected Turkish tobacco plants. Samples of each of the 3 viruses were taken to pH 3.5 by the addition of 0.2 N HCl with vigorous stirring, and each sample was then divided into 2 equal portions. To one portion of a sample of each virus was added an equal volume of 0.001 N HCl containing 2 mg. of crystalline pepsin nitrogen per cc., and to the other portion was added an equal volume of 0.001 N HCl. The hydrogen-ion concentrations of the 3 pepsin-treated preparations and of the 3 controls were found to be pH 3.5 $\pm$ 0.2. Immediately after the addition of pepsin, the 3 preparations were tested for virus infectivity on leaf-halves of *Nicotiana glutinosa* against the 3 controls, and all preparations were then placed in a room at 37° C. They were removed and again tested after 1, 2, 3, 5, and 8 days. The results of the tests are given in table 7. It may be seen that all 3 strains of tobacco-mosaic virus are inactivated on digestion with pepsin. Dilutions of the controls indicate that less than 1 per cent of the virus remains in the virus-pepsin digests after 8 days' incubation at 37° C. The complete inactivation of the masked strain after 8 days' incubation is due, probably, to the fact that its concentration in the original preparation was much less than the concentrations of the other 2 viruses. The results indicate that the action of pepsin on strains of tobacco mosaic is similar to the action on tobacco-mosaic virus itself.

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used in most of the experiments at 37° C., drops away from the line for the controls sharply, thus indicating a rapid inactivation of virus. The line for the results obtained when the pepsin concentration is reduced to 1/10 (C × 1/10) falls off very gradually, whereas the line for the results obtained when the pepsin concentration is increased 10 times (C × 14) falls off almost at right angles. When new or active virus was added to the latter solutions (C × 10) there was but little immediate effect on the infectivity of the added active virus. However on digestion the activity of the added virus decreased as indicated by the extension of the line for the results obtained with pepsin concentration C × 10. The line for the results obtained with the usual pepsin concentration (C × 1) at − 15° C. falls very close to the line for the controls, thus indicating little or no inactivation of virus.

TABLE 7.—*The action of pepsin on different strains of tobacco-mosaic virus*

| | Tested → | At once | After 1 day | After 2 days | After 3 days | After 5 days | After 8 days |
|---|---|---|---|---|---|---|---|
| Purified aucuba mosaic + 1.0 mg. pepsin N per cc. (pH 3.5 ± 0.2) | Average | 100.0[a] | 123.0 | 64.5 | 41.8 | 16.1 | 2.6 |
| | Actual | 165.0[b] | 89.0 | 56.0 | 64.0 | 16.0 | 2.0 |
| Infectious juice masked strain of virus + 1.0 mg. pepsin N per cc. (pH 3.5 ± 0.2) | Average | 76.5 | 99.0 | 56.5 | 45.3 | 10.0 | 0.0 |
| | Actual | 144.0 | 128.0 | 68.0 | 57.0 | 8.0 | 0.0 |
| Infectious juice yellow strain of virus + 1.0 mg. pepsin N per cc. (pH 3.5 ± 0.2) | Average | 81.2 | 94.5 | 53.5 | 50.8 | 37.7 | 8.1 |
| | Actual | 289.0 | 489.0 | 88.0 | 281.0 | 63.0 | 19.0 |

[a] Numbers represent the average number of lesions per half-leaf obtained on 10 or more half-leaves of *Nicotiana glutinosa* with the designated virus-plus-pepsin preparation divided by the average number of lesions per half-leaf obtained on the other halves of the same leaves with the corresponding control preparation, multiplied by 100.

[b] Numbers represent the average of the actual number of lesions per half-leaf obtained on 10 or more half-leaves of *N. glutinosa* on inoculation with the designated virus-plus-pepsin preparation.

## DISCUSSION

The experiments described in this report show that the infectivity of the virus of tobacco mosaic is lost on digestion with pepsin only under conditions favorable for proteolytic activity. There is no appreciable immediate change in the infectivity of the virus on addition of pepsin as measured on *Nicotiana glutinosa* regardless of the hydrogen-ion concentration of the virus-pepsin mixture. This is in striking contrast to the effect caused by trypsin where the addition of even very small amounts of trypsin caused an immediate loss of virus infectivity. Pepsin has no effect on the infectivity of the virus either at once or on standing at pH 7 or pH 8, hydrogen-ion concentrations at which pepsin is inactive as a proteolytic agent. Pepsin possesses practically no proteolytic activity at pH 5 or pH 6, and, likewise, has almost no effect on the infectivity of virus at pH 5 or pH 6. At pH 4 pepsin manifests about 10 per cent of its maximum proteolytic activity and at this hydrogen-ion concentration slowly, without appreciable immediate effect, inactivates the virus. Pepsin has its maximum proteolytic

activity at about pH 2, but it is impossible to carry out experiments at this hydrogen-ion concentration, since the virus is inactivated at pH 2. However, pepsin exhibits over 50 per cent of its maximum proteolytic activity at pH 3, a hydrogen-ion concentration that inactivates virus very slowly and it was found that at pH 3 preparations of both commercial and crystalline pepsin inactivate purified virus and virus in infectious juice very rapidly. Similar results were obtained with aucuba mosaic, and a yellow and a masked strain of tobacco-mosaic virus. The rate of the inactivation was found to be proportional to the amount of pepsin present. No inactivation occurs if the digestion mixture at pH 3 is kept at $-15°$ C., at which temperature pepsin is practically inactive. The fact that pepsin inactivates virus only under conditions that are favorable for proteolytic activity, and that the rate of inactivation varies directly with the pepsin concentration, is evidence that the loss of virus infectivity is due to the proteolytic action of pepsin.

Pepsin-virus digestion mixtures, in which the virus has been inactivated, have no appreciable effect on the infectivity of virus added to such mixtures. This fact indicates that such digestion mixtures are not toxic to virus or to plants susceptible to virus, and, hence, that any active virus present in such mixtures would manifest its infectivity on inoculation to susceptible plants. The failure of pepsin-virus mixtures to produce lesions is, therefore, an indication that no active virus is present.

The infectivity of pepsin-inactivated virus was not regained by heating or by diluting. This fact indicates that the inactivation is not similar to the loss of infectivity caused by the addition of trypsin. The fact that the addition of juice from healthy Turkish tobacco plants or of infectious juice that has been inactivated by heat or by high or low hydrogen-ion concentrations does not reactivate the virus in pepsin-virus digestion mixtures, is an indication that the inactivation is not due to the loss of substances or hydrolysis of proteins present in the above-described preparations. The results indicate, therefore, that the inactivation of tobacco-mosaic virus on digestion with pepsin is due to the proteolytic action of pepsin. Because of the specificity of pepsin, this suggests that the virus is a protein, or closely associated with a protein, that may be hydrolyzed with pepsin.

It is believed that Lojkin and Vinson (6) did not report inactivation of virus on digestion with pepsin because, as they themselves suggest, virus preparations at hydrogen-ion concentrations at which pepsin possesses little or no proteolytic activity were used. Most of their experiments appear to have been carried out at pH 4.7 or above, hydrogen-ion concentrations at which pepsin is almost inactive. One experiment was performed at pH 3.3, but they reported the control was also inactivated. This result

may have been due, not to inactivation of the control, but to the failure of the Turkish tobacco plants, which they used as test plants, to respond to virus at pH 3.3. It has been found that the susceptibility of Turkish tobacco plants to virus is markedly lowered at pH 3, while that of *Nicotiana glutinosa* is but slightly affected. In one experiment with pepsin at pH 3.5, using *N. glutinosa* as the test plant, Lojkin and Vinson found that the pepsin digest gave 89.5 per cent as many lesions as did the control after 2 days' incubation, and only 50.5 per cent as many lesions as did the control after 11 days' incubation. Although they came to the conclusion that pepsin does not attack the virus, it is believed that the decrease in the number of lesions mentioned above in their experiment at pH 3.5 was due to the action of pepsin on the virus.

Caldwell (1) stated that in his experiments he prepared an inoculum from tomato leaf tissue and diluted this 1 to 50. He did not give the hydrogen-ion concentration of his preparation, but if he made the dilution with water and did not otherwise change the hydrogen-ion concentration, the preparation would be near pH 5.8. Since pepsin is practically inactive above pH 4, peptic digestion would not be expected.

## SUMMARY

Pepsin has no appreciable immediate effect on the infectivity of tobacco-mosaic virus or its strains including aucuba mosaic, a yellow and a masked strain at pH 3 to pH 8, inclusive, as measured on *Nicotiana glutinosa*.

Pepsin has no appreciable effect on the infectivity of tobacco-mosaic virus at pH 7 and pH 8 and but little effect at pH 5 and pH 6, even on prolonged digestion at 37° C. Pepsin slowly inactivates this virus at pH 4 on prolonged digestion at 37° C.

Pepsin inactivatives tobacco-mosoic virus, and the strains mentioned above, rapidly at pH 3 and 37° C. Pepsin fails to inactivate virus at pH 3 and a temperature of −15° C., even on prolonged standing.

The rate of inactivation of virus was found to be proportional to the concentration and activity of pepsin, and to the time of digestion.

Pepsin-virus digestion mixtures, containing inactive virus, have no appreciable immediate effect on the infectivity of virus added to such mixtures. This indicates that materials toxic to virus or test plant are not produced on digestion of virus with pepsin.

Attempts to regain the infectivity of virus inactivated by pepsin have resulted in failure.

Since pepsin inactivates virus only under conditions favorable for proteolytic activity, and since the rate of inactivation of virus varies directly with the concentration of active pepsin, it is concluded that the

inactivation of virus is due to the proteolytic action of pepsin. This suggests that the virus of tobacco mosaic is a protein, or very closely associated with a protein, which may be hydrolyzed with pepsin.

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# SCLEROTIAL ROT OF CORN CAUSED BY **RHIZOCTONIA ZEAE**, N. SP.

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In the summer of 1932 several rotted mature ears of corn, *Zea mays* L., were found at Quincy, Florida, that were somewhat similar in appearance to ears rotted either by *Diplodia zeae* (Schw.) Lev. or *D. macrospora* Earle. Certain characteristics, however, indicate that the rot was not caused by either of these fungi. These diseased ears had a somewhat sour fungoid odor, which is different from the dry, musty odor accompanying ears rotted either by *D. zeae* or *D. macrospora*. The outer husks of some of the diseased ears were covered with small sclerotia that are not found on ears rotted by either of the above fungi. Isolations of the fungus were made from these ears and the pathogenicity of the fungus to corn seedlings in the greenhouse and laboratory and to ears of corn in different stages of development in the field has been determined. Studies on the morphology and physiology of the fungus showed that the fungus belonged in the genus Rhizoctonia. Results of these investigations and a description of the disease and the parasite are given herewith.

## SYMPTOMS

Diseased ears are shrunken and often tightly clasped by the husks because of the mycelium of the fungus. In the early stages of infection diseased ears and their husks are covered with the salmon pink mycelium, and in the later stages they have a dull grayish appearance due to the change in color of the mycelium. The outer husks often are covered with small, immature white, as well as mature brown to dark brown, sclerotia attached to the mycelial strands. The mycelium is visible on the kernels and between the kernel rows (Fig. 1, B), as with ears rotted by *Diplodia zeae* or *D. macrospora*, but it may or may not be visible on the kernels of ears that are not severely diseased. On some diseased ears the mycelium can be seen only on the base of the kernels.

## THE CAUSAL ORGANISM

### Isolations

The first isolations were made from kernels of the diseased ears (Fig. 1, B) originally discovered. Individual kernels were taken from the ears and were surface-sterilized in corrosive sublimate 1:1000 for 10 minutes. They were planted in Petri dishes containing potato-dextrose agar (Fig.

2, B). The mycelium grew from the kernels and had covered the entire surface of the medium within 3 days. After 7 days numerous small sclero-

FIG. 1. A. Healthy ear. B. Two ears rotted from natural infection by *Rhizoctonia zeae*.

tia, characteristic of the genus Rhizoctonia, were produced. A large number of isolations were made from such diseased ears and the same fungus was obtained consistently.

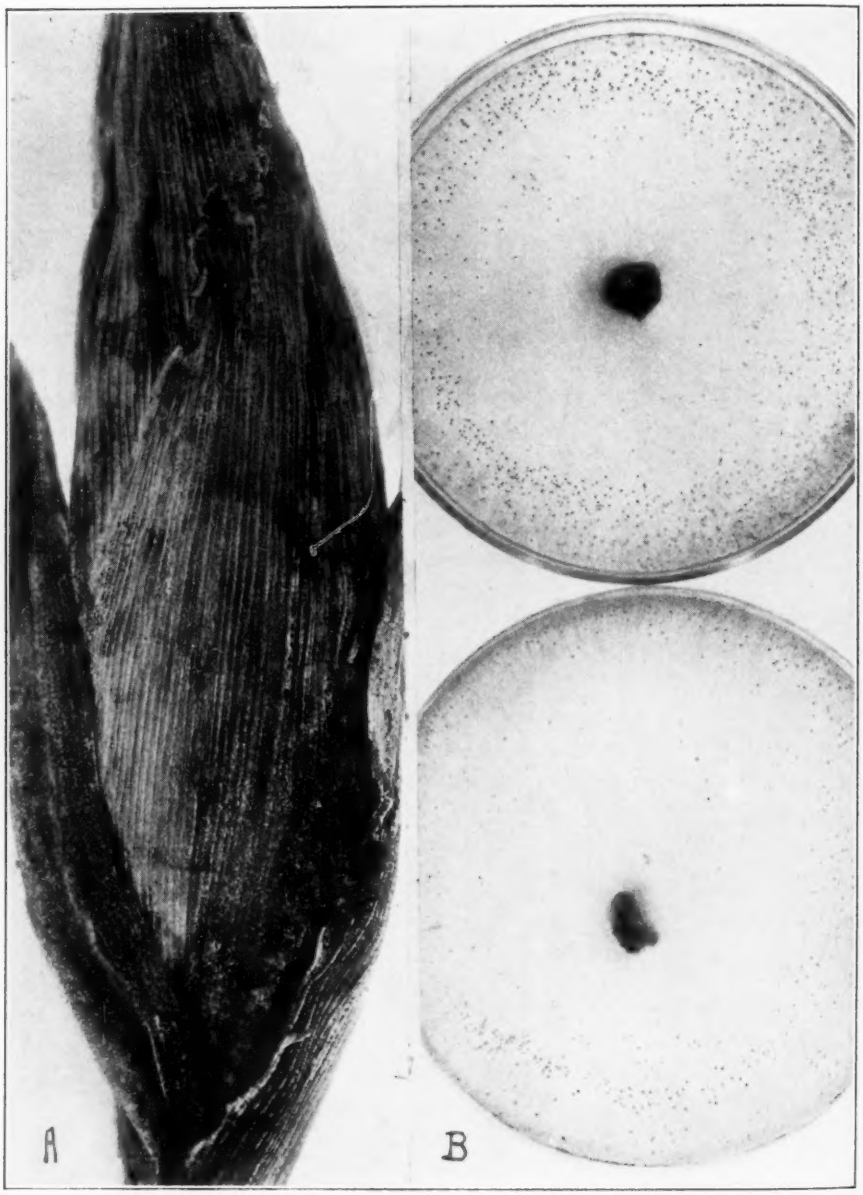


FIG. 2. A. Ear of corn rotted by artificial inoculation of *Rhizoctonia zeae*. Note numerous sclerotia on outer husks. B. Pure cultures of *R. zeae* growing from diseased surface-sterilized kernels on potato-dextrose agar.

## Pathogenicity

*Seedlings.*—Healthy corn seedlings, growing in the greenhouse and in the field, were inoculated with sclerotia and mycelium of the fungus growing on sterilized corn meal. The seedlings were inoculated by placing the

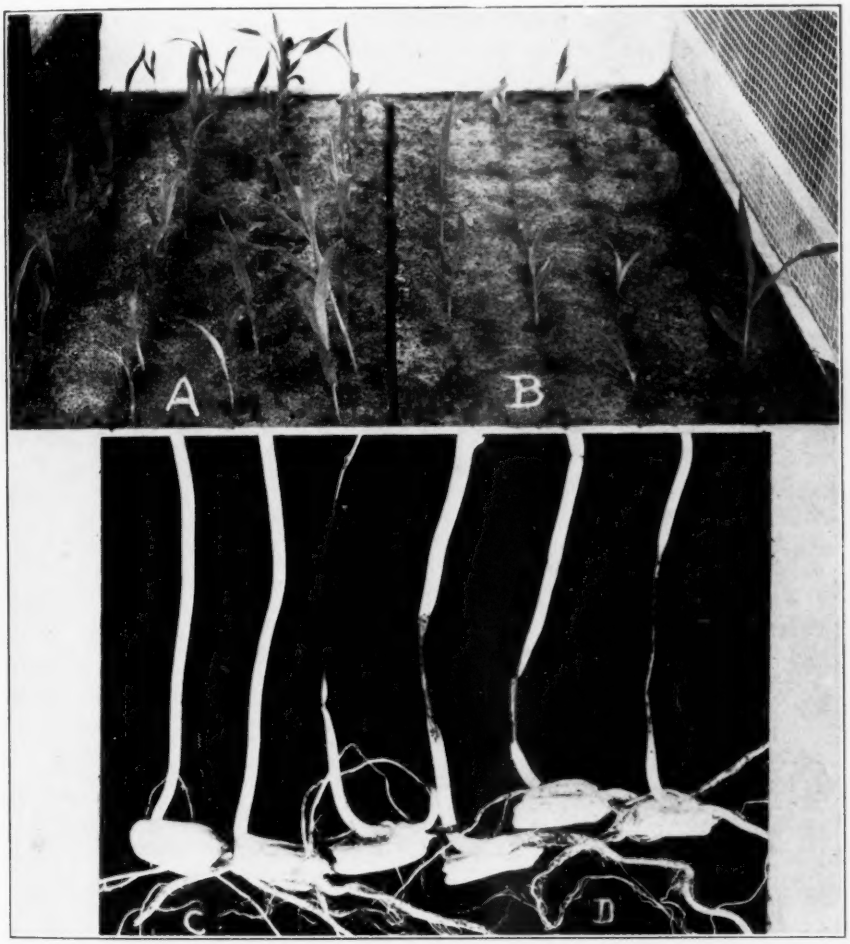


FIG. 3. A and B. Corn seedlings growing in greenhouse in sterilized soil. A. Noninoculated (100 per cent emergence). B. Inoculated with *Rhizoctonia zeae* (31 per cent emergence). C and D. Grown in laboratory in sterilized soil. C. Noninoculated. D. Inoculated with *R. zeae*.

inoculum in the soil around the roots, on the surface of the soil around the stalks, and in incisions in the stalks. The fungus grew readily and produced numerous sclerotia on the surface of the soil and in the incisions in

the stalks, but only slight infection was produced on the nonwounded seedlings.

In another test healthy sterilized kernels were planted in sterilized soil on a greenhouse bench. This planting consisted of a plot of 8 rows, 10 hills per row and 1 kernel per hill. The soil in part of the plot was inoculated by placing a kernel with the fungus growing on it in each hill with the healthy kernel, the corn in the other part of the plot was the check. Only 31 per cent emergence was obtained in the inoculated soil, while 100

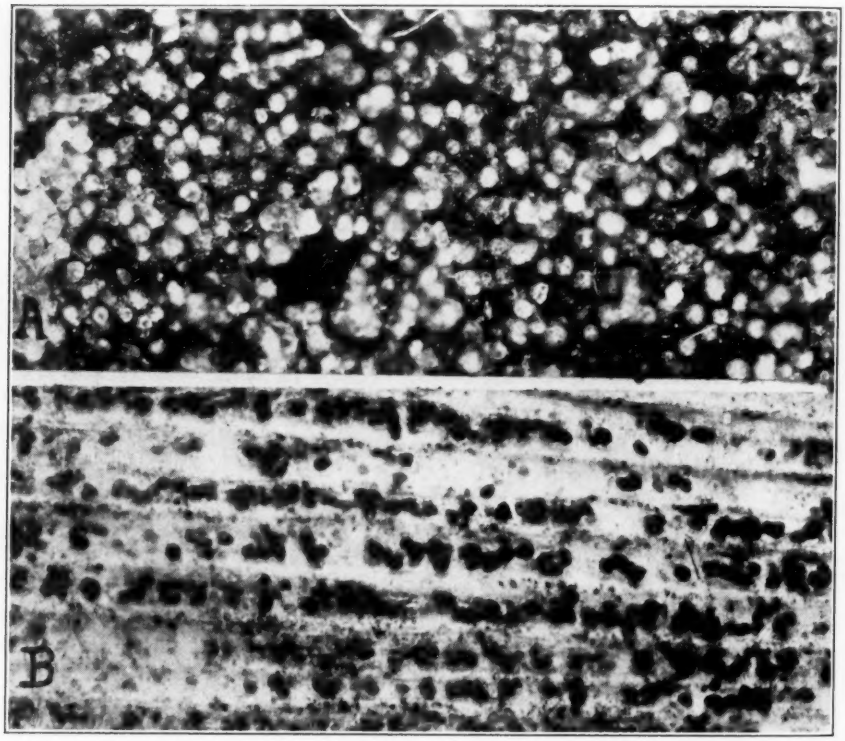


FIG. 4. Sclerotia. A. On surface of inoculated soil. ×7. B. On husk. ×6.

per cent emergence was obtained in the check (Fig. 3, A and B). The fungus either killed the kernels before they germinated or killed the seedlings before they emerged. It failed to kill any of the seedlings after they had emerged. The fungus grew readily in the soil and produced numerous sclerotia on the soil surface (Fig. 4, A).

A test was conducted in the laboratory to study the parasitism of the fungus to corn seedlings under controlled conditions. Twenty healthy sterilized corn kernels were evenly distributed on the bottom of each of 4

crystallization dishes. To 2 of the dishes was added sterilized soil with which inoculum, consisting of the fungus growing on sterilized corn meal, had been mixed. To the remaining 2 dishes, sterilized soil, mixed with sterilized corn meal, was added to serve as checks. Sufficient water was added to each dish to bring the soil up to a desirable moisture content. Each day the dishes were weighed and water was added to bring them to their original weight. The dishes were placed in electrically controlled chambers adjusted to a constant temperature of 30° C. After a period of 9 days, the seedlings were washed free from the soil and observed for infection. Sixty per cent of the seedlings in the inoculated soil were infected, while no infection developed in the checks. The infected seedlings were characterized by a rotting of the mesocotyl at or just below the soil surface (Fig. 3, D), while the roots were nearly free from infection.

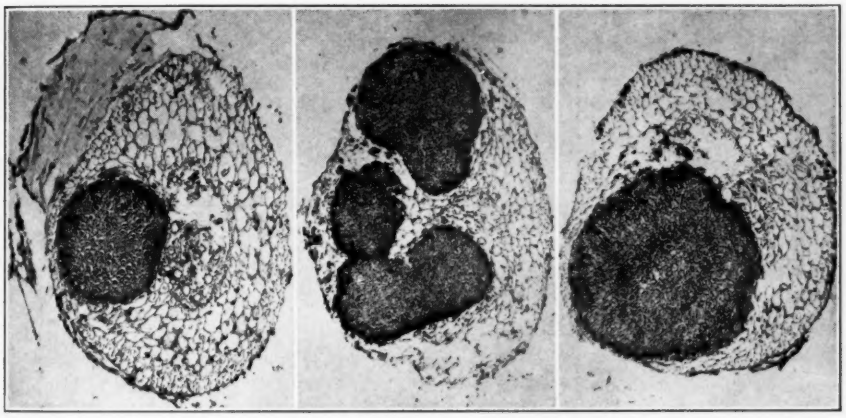

FIG. 5. Cross sections of sclerotia of *Rhizoctonia zeae* of various sizes and shapes in roots of corn seedlings. ×30.

Healthy sterilized kernels also were planted on potato-dextrose agar slants in test tubes and placed in a chamber at a constant temperature of 30° C. After these kernels had germinated the seedlings were inoculated with sclerotia or a small portion of the mycelium. Infection of the seedlings was evident after 3 to 4 days and most of the seedlings were dead at the end of 8 days. The mycelium invaded the seedling roots and mesocotyls, and sclerotia were formed in these parts, as well as on the surface, of the host. Cross sections of some of the roots showing sclerotia of various shapes, and sizes as they are formed in the roots are shown in figure 5.

In the above tests the amount of inoculum probably was in excess of what normally occurs in the field, but the tests indicate the parasitic capabilities of the fungus on corn seedlings.

TABLE 1.—*Comparative susceptibility of ears of two varieties of field corn in different stages of development of Rhizoctonia zeae*

| Date of inoculation | Method of inoculation | Stage of development of ears | Condition of inoculated ears at time of harvesting | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Whatley | | | Cuban Yellow Flint | | |
| | | | No. inoculated | No. with rotted kernels | No. with sclerotia on husks | No. inoculated | No. with rotted kernels | No. with sclerotia on husks |
| June 21 | Inoculum inserted in ear tips | Blister | 25 | 12 | 9 | 20 | 14 | 10 |
| " 30 | " | Early Milk | 25 | 20 | 23 | 22 | 17 | 13 |
| July 6 | " | Milk | 24 | 11 | 6 | 25 | 11 | 6 |
| " 15 | " | Late Milk | 25 | 14 | 14 | 23 | 7 | 6 |
| " 22 | " | Dent | 24 | 4 | 6 | 20 | 3 | 4 |
| June 21 | Inoculum inserted in ear shanks | Blister | 25 | 24 | 18 | 22 | 19 | 19 |
| " 30 | " | Early Milk | 25 | 20 | 24 | 25 | 24 | 23 |
| July 6 | " | Milk | 25 | 25 | 18 | 20 | 15 | 15 |
| " 15 | " | Late Milk | 25 | 22 | 22 | 23 | 9 | 8 |
| " 22 | " | Dent | 21 | 19 | 13 | 25 | 10 | 10 |
| June 21 | Inoculum dropped in crevice between ear and sheath | Blister | 24 | 4 | 6 | 20 | 3 | 4 |
| " 30 | " | Early Milk | 22 | 3 | 4 | 20 | 2 | 3 |
| July 6 | " | Milk | 20 | 3 | 3 | 23 | 1 | 2 |
| " 15 | " | Late Milk | 24 | 2 | 4 | 25 | 1 | 1 |
| " 22 | " | Dent | 23 | 1 | 2 | 20 | 0 | 0 |

*Ears.*—Although the fungus is capable of inhabiting the soil and causing infection of corn seedlings in the soil, observations made thus far indicate that it causes most damage to the ears of the corn plant. Experiments were conducted in the field in which ears of corn of a white dent and a yellow flint varieties were inoculated at different stages of growth. The inoculum consisted of sterilized corn kernels on which the fungus was growing. At intervals of 6 to 9 days from June 21 to July 22, inclusive, 20 to 25 ears were inoculated by each of 3 methods, as follows: (1) Inoculum placed in wound in ear tip; (2) inoculum placed in wound in ear shank; (3) inoculum dropped in crevice between the ear and sheath.

The inoculated ears were harvested August 20 and examined for visible signs of infection. As shown in table 1, infection was obtained by all 3 methods of inoculation. The method producing the highest percentage of infected ears with visible signs of rotting was that in which the inoculum was inserted in the shank. By this method the inoculum was inserted in the moist shank of the ear where it was protected from drying. Ears inoculated by inserting the inoculum into the tips gave a much higher rate of infection than was obtained by dropping the inoculum between the ear and sheath. The inoculum dropped in crevices between the ear and sheath suffered a great deal more from lack of moisture than that inserted in the ear tips.

In these tests ears in the early stages of growth were more susceptible to infection than those in the later stages of development. This was probably because the young, tender ears were easily penetrated and contained more moisture to support the growth of the fungus than older ears that were beginning to dent and harden. The Whatley or white dent was more susceptible to infection than the Cuban yellow flint variety, and especially so as the ears approached maturity.

A majority of the infected ears were invaded and thoroughly rotted by the fungus (Fig. 6), and sclerotia developed on the husks of many of them. The mycelium apparently enters the kernel at its base, since the fungus is first detected in this region. From this point the mycelium grows throughout the kernel and is found in both the endosperm and embryo. The starch grains in the kernels are completely destroyed, thus reducing the weight of the ears. In addition to using up the food stored in the kernels, the fungus completely destroys the viability of the kernels.

## Taxonomy

A study of the literature has failed to reveal reports of a similar fungus attacking corn and especially the ears. Although several species of Rhizoctonia have been reported on corn, all of them appear quite distinct from the species discussed herein. Mackie (1) reported *Sclerotium bataticola*

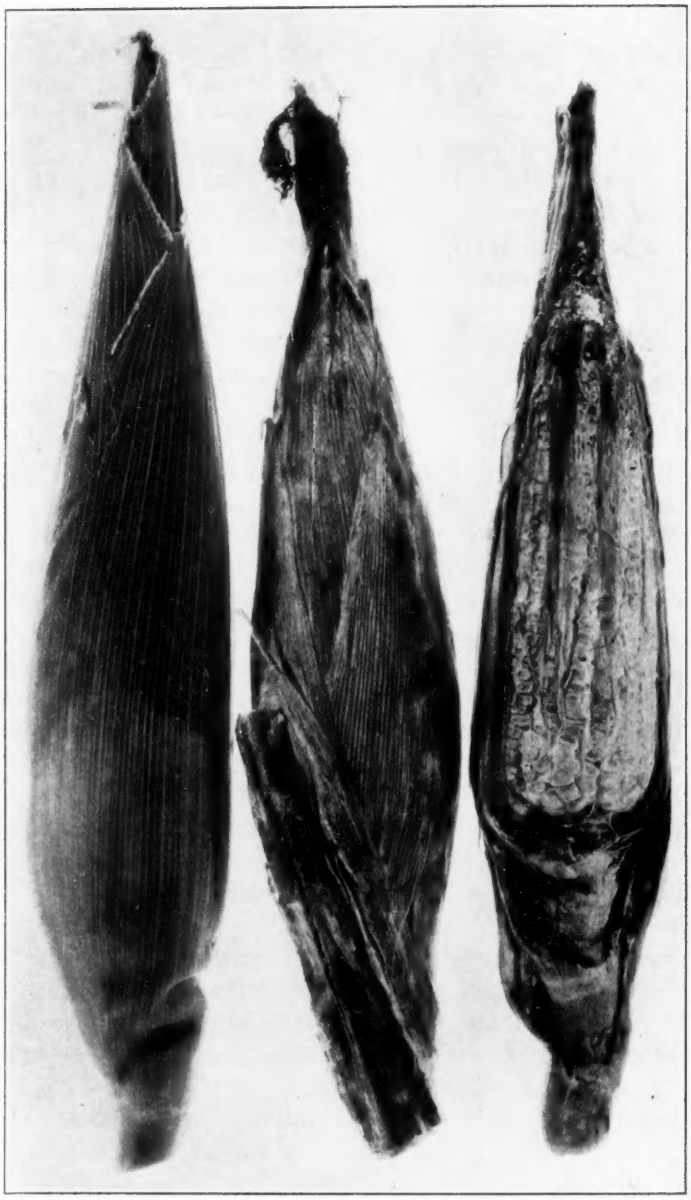

FIG. 6. Ears of corn inoculated in the tip with cultures of *Rhizoctonia zeae*. Healthy ear (left).

Taub. as causing a dry rot of maize, but this dry rot was limited to the pith of the stalk. He (2) later called it charcoal rot, as it was first named by Taubenhaus (6) under the above binomial. This form, bearing only sclerotia, is generally accepted to be *Rhizoctonia bataticola* (Taub.) Butler, as reported by Mackie (2). Peltier (4) reported *Rhizoctonia solani* Kühn on corn roots in the field. Rolfs (5), in 1905, reported *Corticium vagum* B. & C. on sweetcorn in Florida, but did not state on which part of the plant it was found. A somewhat similar Rhizoctonia, but one that produces smaller sclerotia than the one described herein, was reported by Matz (3) on fig in Florida.

Since the disease on corn is undescribed and is caused by a fungus different from any species of Rhizoctonia previously described, it is considered to be a new species. Consequently, the parasitic fungus is described as follows:

**Rhizoctonia zeae**, *sp. nov.*—Sclerotia submerged and superficial in culture, 0.5 to 1.0 mm. in diameter; mostly superficial on host, 0.1 to 0.5 mm. in diameter, white when young, brown to dark brown at maturity; homogeneous in structure and color, hard at maturity, usually single, sometimes conglomerated, produced abundantly on host and in culture; hyphae 4 to 10μ wide; at first, hyaline and granular, reddish-brown in old cultures, salmon-pink on host, later becoming dull gray, multiseptate, constricted at septum.

Produces ear rot of corn, *Zea mays;* sclerotia produced on outer ear husks. (No. 9414—Fla. Agr. Exp. Sta. Herb.).

## Morphology and Physiology

The general morphological characteristics of the above-described fungus indicate that it belongs to the genus Rhizoctonia. It has not yet been connected with a perfect stage. In young cultures the hyphae ramify profusely; the branches first appear to be more or less parallel to the main axis and constricted at the base, which is characteristic of the genus Rhizoctonia. Later, the hyphae branch irregularly, usually at right angles to the parent hyphae, and become multiseptate. The mycelium grows rapidly, covering the surface of media in a 9 cm. Petri dish within 3 days. After 7 days numerous sclerotia are embedded in the medium, and on the surface (Fig. 2, B). The origin and development of the sclerotia were studied and different stages are shown in figure 7. Although the formation of a sclerotium may begin from a single hypha, it appears to start usually from an aggregation of hyphae. By repeated branching and anastomosing of these hyphae, it soon becomes a dense mass with short hyphae protruding and eventually a mature sclerotium (Fig. 7, D). Cross

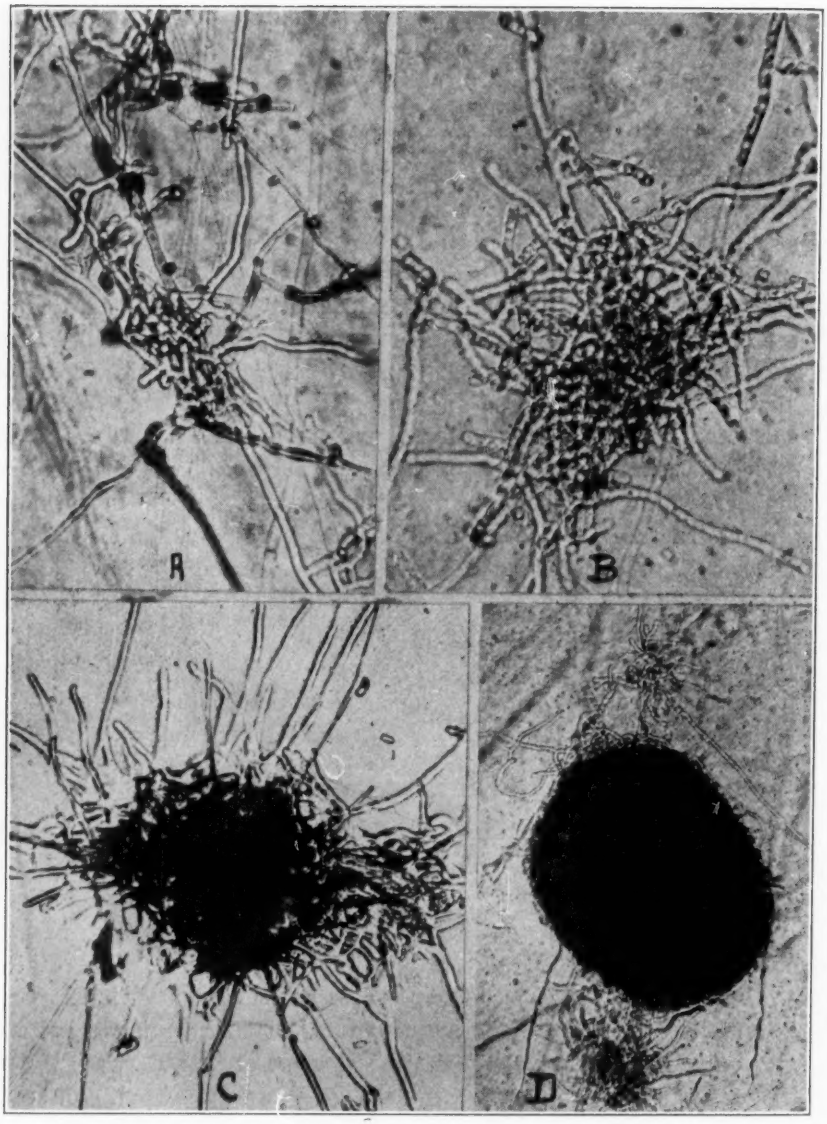


FIG. 7. Stages in the formation of a sclerotium of *Rhizoctonia zeae*. A. Small mass of entangled hyphae in the early stage. ×225. B. Later stage in the same. ×225. C. Later stage with branches protruding, some of which are rebranching. ×175. D. The network rounded into a mature sclerotium, with sclerotia beginning to form on each side apparently on the same parent hyphae. ×60.

sections of mature sclerotia (Fig. 5) show that they consist of interwoven hyphae, resulting in a homogeneous structure.

At the end of a year, pure cultures of the fungus were still viable. Sclerotia of the organism were transplanted for several generations and the original characters of growth, form, and size of sclerotia were maintained throughout the period.

*Relation of Temperature to Mycelial Growth in Culture.*—The culture of the organism used in these studies was isolated from corn ears and had been grown in pure culture on potato-dextrose agar. A series of 60 plates of potato-dextrose agar were inoculated in the center with uniform bits of mycelium from a 3-day-old culture, and 5 plates were incubated for 3 days at each of 12 different temperatures ranging from 11 to 42° C. At the end of 3 days the diameters of the colonies were measured and the average diameter of the 5 plates at each temperature is presented in table 2.

TABLE 2.—*The relation of temperature to the mycelial growth of Rhizoctonia zeae on potato-dextrose agar*

| Temperature in degrees Centigrade | 11 | 14 | 17 | 21 | 24 | 27 | 30 | 33 | 36 | 38 | 40 | 42 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Average diameter of colonies in centimeters | 0 | .7 | 1.2 | 3.6 | 6.1 | 6.6 | 7.1 | 7.8 | 6.1 | 3.4 | .3 | 0 |

The mycelium at the lower temperatures (14 to 21° C.) was extremely thin. Colonies of approximately the same diameters at higher temperatures were more dense. At temperatures approaching the optimum and at the optimum the mycelium was dense and whitish; later the cultures became dotted with sclerotia as shown in figure 2, B. In old cultures the mycelium becomes reddish-brown. The optimum temperature for growth in this test was close to 33° C., the minimum between 11° and 14° C., and the maximum between 40° and 42° C. The fact that the fungus grew at 38° and slightly at 40° C. will account for its ability to thrive and cause infection of corn ears that usually are exposed to the direct sunlight and high air temperatures prevalent during the summer. Sclerotia stored in an electric oven at a constant temperature of 50° C. were still viable after 60 days. This temperature is much higher than that usually occurring in the field, and shows that the fungus is thoroughly capable of tolerating the high summer temperatures.

*Relation of Hydrogen-ion Concentration to Mycelial Growth.*—*Rhizoctonia zeae* was grown on a 2 per cent potato-dextrose agar adjusted to 12 different hydrogen-ion concentrations. Five test tubes, each containing 15 cc. of this medium, were used for each concentration, and the concentra-

tions used ranged from pH 2.5 to 9.0. The adjustments were made by adding the required number of drops of HCl and NaOH of different normalities, the determinations being made with a Youden Hydrogen-ion Concentration Apparatus. After the medium was adjusted, it was poured into Petri dishes, inoculated in the center with uniform bits of mycelium, and exposed to a constant temperature of 33° C. At the end of 3 days the diameters of the colonies were measured, and the average diameter of the 5 plates at each concentration is presented in table 3.

TABLE 3.—*The relation of hydrogen-ion concentrations of potato-dextrose agar to the rate of mycelial growth of Rhizoctonia zeae at 33° C.*

| pH reaction | 2.5 | 3.3 | 3.9 | 4.6 | 5.1 | 5.5 | 6.2 | 6.8 | 7.4 | 8.2 | 8.6 | 9.0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Average diameter of colonies in centimeters | 0 | 1.8 | 3.2 | 5.6 | 6.8 | 7.4 | 7.6 | 8.0 | 7.2 | 4.6 | 3.2 | 2.2 |

The results show that the pH reaction most favorable for growth was 6.8, which probably is very near the optimum. The organism grew slowly at pH 3.3 and was inhibited at pH 2.5. The maximum reaction for growth was not obtained but it is probably not much higher than pH 10.0, because growth was slow at pH 9.0.

## Overwintering

Since the fungus does not grow very readily at the lower temperatures, it seems probable that it remains more or less inactive during the winter, depending on moisture and temperature conditions. The fungus overwinters in the form of sclerotia, scattered throughout the soil and in the infected refuse left in cornfields. This also probably aids in dissemination, since the sclerotia may be washed considerable distances across fields. The organism may also live over as dormant mycelium in the infected kernels left in the field. Sclerotia placed in small glass vials and sealed remained viable for 25 days at 0° C., and sclerotia frozen in a cake of ice remained viable for 15 days. Such low temperatures do not occur in Florida in the field, but the above test illustrates the ability of the fungus to tolerate low temperatures and thus remain viable over winter in the field.

## SUMMARY

A sclerotial dry rot of corn ears in the field is described for the first time. This disease is caused by the fungus *Rhizoctonia zeae*, n. sp. At certain stages it could be easily mistaken for that caused by *Diplodia zeae* or *D. macrospora*. The outstanding symptoms of the disease are discussed.

The fungus is capable of rotting the seedling roots and mesocotyls of corn, but is most important as causing an ear rot. Ears of corn in the early stages of growth proved more susceptible to infection than in the later stages of development.

The morphological characters of the fungus were studied, including the origin and development of the sclerotia.

*Rhizoctonia zeae* grows well and produces numerous sclerotia on standard culture media and on sterilized corn kernels and corn meal.

In pure culture, on potato-dextrose agar, the minimum temperature for mycelial growth for the organism is between 11 and 14° C., the maximum lying between 40 and 42° C. and the optimum near 33° C.

No growth developed on potato dextrose agar at pH 2.5, and the fungus grew best at pH 6.8. The maximum reaction was not determined but it is probably not much higher than pH 10.0.

The fungus hibernates as dormant mycelium and sclerotia in seed and in old plant débris and in the soil.

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# NOTES ON THE CONTROL OF TRANSIT AND STORAGE DECAYS OF TOMATOES BY THE USE OF CHEMICAL WASHES

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## INTRODUCTION

The substantial losses sustained each year by growers and shippers of tomatoes, due to decays developing during transit and storage, have been pointed out by several investigators. In 1918 Shear (7) gave an excellent résumé, with citations to literature, of the losses involved in the fruit and vegetable industry. Rosenbaum (5, 6) and Ramsey and Bailey (2, 3, 4) in subsequent papers have developed and clarified certain phases of the problem as it applies to the tomato industry. Recently, Stevens and Nance (8) have called attention to freight-claim statistics for 1930, which show that "tomatoes stood first in average amount of loss per car lot, with $40.04 as compared with an average for all commodities of $11.25."

While stationed in the winter tomato-shipping section of southern Florida, during the winters of 1929 and 1930, the writer conducted a series of experiments to test the efficacy of a number of chemical solutions or treatments, used as washes, in reducing tomato-fruit transit and storage decays. As circumstances have made it impracticable for the writer to continue these studies, the data accumulated during the 2 seasons are here briefly presented.

## PURPOSE OF EXPERIMENTAL WORK

The purpose of these experiments was to determine the comparative effectiveness of a group of chemical washes in controlling transit and storage tomato-fruit rots, as they occurred on large commercial samples of Florida winter tomatoes. In order that these washes might be tested at the same time for control of specific fruit-rot organisms each sample was inoculated with a pure culture of one of three tomato fruit-rot organisms that cause a large proportion of tomato-fruit decays, namely, *Phoma destructiva* Plowr., *Rhizoctonia solani* Kühn or *Phytophthora terrestris* Sherb.

## EXPERIMENTAL METHODS

The procedure followed in all groups of tests was essentially the same. For each group of treatments mature green tomatoes, as received from the field at the packing house, were carefully sorted and all fruits showing breaks in the skins or any indications of infection were discarded. From

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this quantity of carefully selected and cleaned fruits, individual samples of 25 fruits each were counted out for subjection to the various treatments. Each sample was placed in a 4-quart carrier, where it remained during the course of treatment and storage. All except the noninoculated check fruits were then inoculated with a pure culture of either Phoma, Rhizoctonia, or Phytophthora. The fruits were sprayed with an aqueous spore suspension of the Phoma culture, while the Rhizoctonia or Phytophthora cultures were applied by sprinkling the fruits with an aqueous suspension of the shredded fungous mat. In order to allow the inoculum time to adhere to the fruits the inoculated tomatoes were not placed in the washing solutions until after the free water had evaporated from the fruit surfaces. The washes were held in wooden vats and were maintained at temperatures between 115° and 122° F. while in use, as preliminary tests with most of these washes indicated that they were more effective when used hot, and no apparent injury to the fruits occurred at a maximum temperature of 122° F. Fifty fruits (2 4-quart carriers) were submerged in each wash for 3 minutes. Half the fruits of each lot were then stored without rinsing, the other half were rinsed in tap water before they were placed in storage. In each experiment 50 fruits were stored noninoculated, as they were received from the field, and 50 inoculated fruits were stored without any washing treatment as controls. All fruits were held throughout the period of storage on shelves in one storage room.

The storage room was insulated with a 4-inch layer of sawdust over the ceiling, between the sidewalls, and on the floor. An atomized spray of water was maintained in the center of the room and the air was kept stirred by an oscillating fan during the incubation period. No attempt was made to control the temperature of the storage room, but the air-temperature readings of the room were recorded daily for each experiment. The lowest mean temperature at which any wash was tested was 63.9° F. and the highest mean temperature of any experiment was 74.9° F. The temperature fluctuations for all experiments under consideration were, therefore, not excessive, and, since the various treatments were repeated at different times, the mean storage temperatures of the various treatments (Table 1), averaged about 70° F. where 6 or more tests were made.

The relative humidity of the atmosphere of the room was determined in the morning and evening by means of a sling psychrometer. The mean minimum relative humidity for all experiments was 93.3 per cent and the maximum approached the saturation point. The humidity was quite uniform for all experiments and the range closely approximated the daily fluctuations. The humidity of the storage room was maintained at a higher percentage than tomato fruits would normally encounter in transit and storage, in order to favor the development of the various storage decays and to shorten the storage period.

The tomatoes were held in storage from 9 to 17 days, depending on the rapidity with which the decays attacked the fruits, the usual period being 2 weeks. When the storage period was terminated each fruit was examined and the number of fruits having lesions caused by the fungus with which they were inoculated at the beginning of the storage period was recorded separately. The fruits having lesions caused by other organisms were grouped together. Of course, an indeterminate number of lesions in both groups were initiated in the field, but this does not materially affect the validity of the results, since all treatments and controls in any one group of determinations were subjected to the same field conditions.

## PRESENTATION OF RESULTS AND DISCUSSION

During the 2 seasons this work was in progress, 18 series of tests were conducted in which 8375 tomato fruits were inoculated and treated with 21 different chemical washes. An additional 2200 fruits were used for controls. Table 1 shows the mean percentage of sound fruits obtained in all inoculated rinsed and nonrinsed series at the end of the storage periods by the use of each washing treatment together with the mean percentage of sound fruits found in the corresponding control lots and the mean difference in percentage between them (columns 3, 4 and 5, respectively). The odds of significance shown were computed according to Student's (9, 10) method. In columns 1 and 2 are given the number of tests made with each chemical wash and the mean storage temperature at which all the tests were made.

It will be observed that the odds are significant in every case and are very high for every treatment tested 10 or more times. The average difference in percentage of sound tomatoes over the controls was the highest in those tests where formaldehyde-borax solution was used. An average difference of 59.5 per cent of sound fruit was obtained in the 16 tests. However, the percentage differences of sound fruit obtained with several other washes were nearly as high. The very poisonous mercuric chloride solution was included as a reference point only for obtaining an estimate of the efficacy of the numerous washes of unknown fungicidal and bactericidal value. The average percentage difference of sound fruits obtained with mercuric chloride over the control series was as good as but no better than the best of the nonpoisonous treatments, which indicates that, under these conditions, especially favorable for the development of rot organisms, there is a 45 to 60 per cent decrease in the number of fruits actually infected after treatment with the most effective of the washes used.

The time the fruits are submerged in the wash must of necessity be short for commercial use, since large quantities of fruit must be handled rapidly. Apparently, the infections that have been initiated in the field

TABLE 1.—*Tabulation of the mean percentages of sound tomatoes found at the end of the storage periods of all tests made of each chemical wash as compared with its inoculated controls*

| Washing treatments | Number of tests | Mean storage temperature of all tests | Mean percentages of sound fruits at end of storage periods | | | |
|---|---|---|---|---|---|---|
| | | | Nontreated inoculated control fruits | Inoculated treated fruits | Difference | Odds |
| 1 to 300 commercial formaldehyde[1] $CH_2O$ | 32 | 70.4 | 19.7 | 73.1 | 53.4 | over |
| 1 per cent potash alum $K_2Al_2(SO_4)_4 \cdot 24H_2O$ | 18 | 72.1 | 20.4 | 70.6 | 50.2 | 10,000 to 1 |
| 1 per cent sodium alum $Na_2Al_2(SO_4)_4 \cdot 24H_2O$ | 6 | 70.0 | 26.0 | 74.7 | 48.7 | do |
| 1 per cent ammon. alum $(NH_4)_2Al_2(SO_4)_4 \cdot 24H_2O$ | 8 | 67.6 | 23.6 | 39.5 | 15.9 | 107 to 1 |
| 1 per cent borax $Na_2B_4O_7 \cdot 10H_2O$ | 20 | 70.4 | 20.0 | 55.3 | 35.3 | 74 to 1 |
| 1 per cent chloride of lime $Ca(Cl\,O)_2$ | 22 | 70.9 | 19.5 | 62.0 | 42.5 | over |
| ¼ per cent laundry bleach NaCl O | 6 | 68.8 | 24.0 | 72.0 | 48.0 | 10,000 to 1 |
| ⅛ per cent calomel $Hg_2Cl_2$ | 6 | 68.8 | 24.0 | 71.3 | 47.3 | do |
| ⅛ per cent corrosive sublimate $HgCl_2$ poison | 12 | 71.3 | 21.7 | 79.0 | 57.3 | 263 to 1 |
| 1 per cent borax and 1 per cent potash alum | 16 | 70.6 | 21.0 | 50.0 | 29.0 | 227 to 1 |
| 1 per cent chloride of lime and 1 per cent potash alum | 16 | 70.6 | 21.0 | 78.3 | 57.3 | over |
| 1 per cent chloride of lime and 1 per cent borax | 16 | 70.6 | 21.0 | 76.3 | 55.3 | 10,000 to 1 |
| 1 per cent chloride of lime, 1 per cent borax, and 1 per cent potash alum | 16 | 70.6 | 21.0 | 74.8 | 53.8 | do |
| 1 per cent potash alum in 1 to 300 commercial formaldehyde | 22 | 70.5 | 22.4 | 79.3 | 56.9 | do |

TABLE 1—(*Continued*)

| Washing treatments | Number of tests | Mean storage temperature of all tests | Mean percentages of sound fruits at end of storage periods | | | |
|---|---|---|---|---|---|---|
| | | | Nontreated inoculated control fruits | Inoculated treated fruits | Difference | Odds |
| 1 per cent ammonium alum in 1 to 300 commercial formaldehyde | 4 | 63.9 | 31.0 | 80.0 | 49.0 | 31 to 1 |
| 1 per cent borax in 1 to 300 commercial formaldehyde | 16 | 70.6 | 21.0 | 80.5 | 59.5 | over 10,000 to 1 |
| 1 per cent chloride of lime in 1 to 300 commercial formaldehyde | 16 | 70.6 | 21.0 | 75.0 | 54.0 | do |
| 1 per cent potash alum and 1 per cent borax in 1 to 300 commercial formaldehyde | 10 | 73.0 | 16.8 | 62.4 | 45.6 | do |
| 1 per cent chloride of lime and 1 per cent borax in 1 to 300 commercial formaldehyde | 10 | 73.0 | 16.8 | 71.6 | 54.8 | do |
| 1 per cent chloride of lime and 1 per cent potash alum in 1 to 300 commercial formaldehyde | 4 | 74.9 | 25.0 | 76.0 | 51.0 | 61 to 1 |
| 1 per cent chloride of lime, 1 per cent borax and 1 per cent potash alum in 1 to 300 commercial formaldehyde | 16 | 70.6 | 21.0 | 79.5 | 58.5 | over 10,000 to 1 |

[1] One part of 40 per cent commercial formaldehyde to 300 parts tap water by volume. All the other chemicals were used on a weight-volume basis.

before the tomatoes are picked determine to a considerable extent the keeping quality of tomatoes in storage, whether they are surface sterilized or not before the storage period. Wardlaw and McGuire (11), in Trinidad, indicate that the length of time the different lots of tomato fruits remained sound in their storage experiments was influenced greatly by the incidence of infections under field conditions before the fruits were placed in storage. In this connection, the relative amounts of decay produced during these experiments by the 3 fungi, which cause a large proportion of Florida tomato fruit rots, seem worthy of notice. The comparative percentages of decay caused by Phoma, Rhizoctonia, and Phytophthora in the inoculated and noninoculated controls and also in the series of all experiments treated with commercial formaldehyde are listed in table 2.

TABLE 2.—*Comparison of the percentages of fruits decayed by three tomato fruit-rot fungi in the inoculated and in the noninoculated controls and in the inoculated series submerged in formaldehyde solution*

| Fungus | Inoculated controls Average decay | Noninoculated controls Average decay | Inoculated formaldehyde-treated series Average decay |
|---|---|---|---|
| | *Per cent* | *Per cent* | *Per cent* |
| *Phoma destructiva* | 39.3 | 32.0 | 11.50 |
| *Rhizoctonia solani* | 55.1 | 1.6 | 1.25 |
| *Phytophthora terrestris* | 96.0 | 5.6 | 1.20 |

In interpreting these percentages one must keep in mind that they were derived from results obtained with random samples of fruit from 2 crop years and that all 3 fungi were not tested simultaneously but usually at successive intervals of a few days. However, each fungus was tested several times during the 2 seasons. Remembering that each fruit was selected macroscopically as being sound at the beginning of the storage period, note that a relatively high percentage of Phoma infections occurred in the noninoculated controls, which indicates a high prevalent incidence of undetectable Phoma infections on the fruits as they arrive at the Florida packing houses. On the other hand, the average percentage of infection occurring in the series inoculated with Phoma spores at the beginning of the storage period is but little higher than the noninoculated series, which indicates that the source of most of the Phoma infections that developed during the storage period must be in the field. These results agree with the negative results obtained by Miss Jamieson (1), when she inoculated tomato fruits having unbroken skins that had not previously been exposed to infection by Phoma. It seems evident, however, that during development under

conditions that prevail in Florida tomato fields, Phoma becomes sufficiently embedded in the tissues of the stem region of many apparently unblemished tomato fruits to enable it to produce macroscopic lesions as they approach ripeness. It was also significant that a large number of Phoma lesions developed despite the best of the washing treatments tested.

Although soil rot, *Rhizoctonia solani*, and buckeye rot, *Phytophthora terrestris*, also known as water spot, were periodically very prevalent in the fields during the 2 seasons that the random samples of tomato fruits were tested, it is apparent that the field-infected fruits were more readily segregated from the noninfected tomatoes, as shown by the low average percentage decay caused by these fungi in the respective noninoculated control series. The most probable reason for this seems to be the rapidity with which lesions of these fungi develop after infection takes place as contrasted with the apparently imperceptible development of Phoma until the tomato fruit approaches the ripening stage.

A comparison of the rinsed and nonrinsed series of all washes in all experiments for the 2 years indicates that rinsing the fruits in tap water immediately after they were removed from the washes slightly decreased the average percentage of sound fruits found at the end of the storage period. The difference obtained was, however, insignificant for all washes except those containing formaldehyde in combination with other chemicals, where a small significant difference was obtained in favor of the nonrinsed series (Table 3).

TABLE 3.—*Comparison of the percentages of sound tomato fruits found at end of storage periods for all the rinsed and the nonrinsed series of grouped washes*

| Groups of washes | Nonrinsed series, sound fruits | Rinsed series, sound fruits | Difference, sound fruits | P.E.M. |
|---|---|---|---|---|
| | *Per cent* | *Per cent* | *Per cent* | |
| Washes containing formaldehyde in combination with other chemicals | 79.03 | 69.65 | 9.38 | ± 1.40 |
| Washes containing formaldehyde only | 77.85 | 74.46 | 3.69 | ± 1.26 |
| Washes containing no formaldehyde | 67.43 | 63.62 | 3.81 | ± 3.54 |

Notwithstanding the small consistent differences in favor of the nonrinsed series of all 3 groups of washes it seems inadvisable to recommend leaving the washes on the fruit surfaces unless the advantage is more conclusively shown by further investigation. Although there was no apparent injury to the tomatoes due to any of the washing treatments as used in

the tests recorded in table 1, and they ripened normally with the controls, the nonrinsed fruits would have to have the residues left by the chemical washes removed before they could be marketed.

From time to time interested persons have expressed the opinion that treating tomato fruits with ethylene gas improved their keeping qualities. In order to test this opinion, groups of tomatoes inoculated with Phoma, Rhizoctonia, and Phytophthora and one noninoculated group were kept for 3 successive days in a room where a cubic foot of ethylene gas per 1000 cubic feet of air was liberated each day. Nongassed inoculated and noninoculated groups of tomatoes were kept in an adjoining room for 3 days. All gassed and nongassed groups were then transferred to the storage room. The percentages of decay occurring at the end of 2 weeks in each group of 50 fruits each are recorded in table 4.

TABLE 4.—*Effect of exposure to ethylene gas for 3 days on the development of tomato fruit decays*

| Infecting fungus | Decay in groups not treated with ethylene gas | Decay in groups treated with ethylene gas |
|---|---|---|
| | *Per cent* | *Per cent* |
| Phoma | 20 | 25 |
| Rhizoctonia | 78 | 80 |
| Phytophthora | 100 | 100 |
| Noninoculated | 42 | 36 |

It is apparent that the ethylene treatment had no significant effect on the development of tomato fruit rots in any of these groups.

## SUMMARY

The relative effectiveness of a group of chemical washes in inhibiting tomato fruit rots was tested on commercial samples of "green-wrap" Florida winter tomatoes in storage. Submerging inoculated tomato fruits for 3 minutes in hot washes before placing in storage reduced the number of decayed fruits found at the end of the storage periods an average of 29 per cent for the least effective wash, and an average of 59.5 per cent for the most effective wash used. Rinsing of the fruit immediately after treatment did not significantly increase the number of fruits developing rots in storage, except in the groups of fruit washed in formaldehyde solutions containing other chemicals, where there was a small significant increase in percentage of decayed tomatoes. Most of the Phoma infections that developed on the tomato fruits while in storage were natural infec-

tions that could not be detected when the tomatoes were stored, and part of them could not be inhibited by the most effective washes tested. Ethylene gas as used for accelerating the coloring of tomato fruits had no effect on the development of tomato fruit rots.

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## PHYTOPATHOLOGICAL NOTES

*The Antiquity of Puccinia Graminis and Ustilago Tritici in California.*—In 1880 Harkness and Moore[1] published a list of some 860 species of fungi that they had collected mainly in California in 1876, 1877, 1878, and 1879, and their inclusion of *Puccinia graminis* Pers., in this list, recorded as occurring "On wheat and sugarcane.[2] San. Fran. and S. Is.[3]", is thought to constitute the earliest definite record of that organism in the Pacific Coast region. *Ustilago tritici* (Pers.) Rostr. does not appear in this list and is thought to have been first collected in the Pacific Coast region at a still later date. It is our purpose in publishing this note to call attention to new evidence regarding the antiquity of these 2 organisms in California and to suggest the wider applicability of the method we have employed to the study of the history of parasitic fungi in general in the region under consideration.

Since 1925, Hendry,[4, 5] has given attention to the fragments of higher plants that are a common constituent of sun-dried earthen bricks composing the walls of historic adobe buildings, and by this means has established the identity of numerous wild and cultivated plants, in the localities studied, that were present concurrently with the erection of such buildings. Recently, in the pursuit of these studies, it has become apparent that such higher plants have also occasionally served as hosts for fungi, and that occasionally such organisms may be specifically identified. In the examination of a collection of such plant remains, consisting principally of wheat straw taken from the interior of sun-dried earthen bricks collected from the ruins of the church building of the Mission Nuestra Senora De La Soledad, erected in the central Salinas Valley of California during the years 1793 and 1794, but rebuilt in 1832, following destruction by flood, one of us (Hansen) has identified both *Puccinia graminis* Pers. and *Ustilago tritici* (Pers.) Rostr. Teliospores and urediospores of the former, on both the culm and rachis of Little Club wheat, *Triticum compactum humboldtii,* Kcke., and spores of the latter lodged in the tufts of rachis hairs of the above named wheat.

[1] Harkness, H. W. and J. P. Moore, "Catalogue of the Pacific Coast Fungi." California Academy of Sciences, unnumbered pamphlet, Feb. 2, 1880.

[2] Sorgo, *Andropogon Sorghum* var. *saccharatus,* Alef., was under trial in California in the late 70's and early 80's as a possible source of sugar and was then commonly referred to as "Chinese Sugar Cane" or as "Sugar Cane."

[3] May refer to Sandwich (Hawaiian) Islands, in which case the host was probably true sugar-cane, *Saccharum officinarum,* L.

[4] Hendry, G. W. and M. P. Kelly. The plant content of adobe bricks, Quart. Calif. Hist. Soc. 4: 361–373. 1925.

[5] Hendry, G. W. The adobe brick as a historical source. Reporting further studies in adobe brick analysis. Agr. Hist. 5: 110–127. 1931.

These finds prove the existence of these organisms on the host mentioned in the vicinity of Soledad in the year 1832, antedating by more than 40 years all records known to us for the occurrence of these organisms in California.

We are now studying older material from this and other historic edifices that promises to yield a further extension of our knowledge of the history of pathogenic fungi and their hosts in this region.—G. W. HENDRY and H. N. HANSEN, University of California, Berkeley, California.

*Some Pathological Observations on Sugar Cane × Sorghum Hybrids in Florida.*—The first cane × sorghum hybrids produced in Florida were in 1931–1932,[1] when the sugar cane variety POJ.2725 was crossed successfully with Texas Seeded Ribbon and Early Orange, varieties of *Holcus sorghum* var. *saccharatus* Bailey. POJ.2725 was first crossed successfully with *Sorghum durra* Stapf by Thomas and Venkatraman[2] in India. While mention has been made of various morphological and structural characters of leaves, stems, and flowers of the resulting $F_1$ progeny of this cross, there is no published record of the occurrence of diseases among the hybrids.

During a detailed study of the inheritance of certain leaf, stem, and floral characters among 58 hybrids between POJ.2725 and Texas Seeded Ribbon sorghum, notes also were made of the occurrence on them of mosaic and certain common leaf and sheath diseases frequently attacking sugar cane and sorghum.

Although POJ.2725 has been reported to show occasional infection with mosaic in certain regions of Cuba,[3] it has never been known to become infected with this disease in Florida, even with thousands of acres under commercial culture and exposed to infection. POJ.2725 is nearly immune from red rot of the sheath (*Colletotrichum falcatum* Went.) but, depending on soil and location, it is, however, more or less susceptible to eye spot (*Helminthosporium ocellum* Faris), brown stripe (*Helminthosporium stenospilum* Drechsler) and brown spot (*Cercospora longipes* Butler).

While the sorghum variety, Texas Seeded Ribbon, has frequently been found infected with mosaic when growing very close to the hybrid plots, it never has been found infected with any of the other diseases occurring on sugar-cane leaves or sheaths. One of the commonest diseases present on the leaves of this sorghum host was the leaf rust (*Puccinia purpurea* Cooke).

[1] Bourne, B. A. Cane breeding experiments. Florida Agr. Exp. Sta. Ann. Rpt. 1932: 205–207. 1932.

[2] Thomas, R. and T. S. Venkatraman. Sugarcane-sorghum hybrids. Agr. Jour. Ind. 25: 164. 1930.

[3] Stahl, C. F. and James A. Faris. The behavior of the new POJ canes in relation to sugar cane mosaic in Cuba. Trop. Plant Res. Found. Bul. 9: 1929.

A summary of observations to date on the susceptibility of the hybrid progeny to 4 diseases shows that approximately two-thirds of the hybrids are susceptible to the 2 Helminthosporium diseases of sugar cane, one-third to brown spot (*Cercospora longipes*) and one-fourth to the red rot of the sheath (*Colletotrichum falcatum*).

So far, none of the progeny of the above cross or any other hybrids between POJ.2725 and varieties of *Holcus sorghum* var. *saccharatus* have been found infected with mosaic. However, the results of 2 years' observations should not be considered final, as certain varieties of canes that are normally immune from mosaic on Okeechobee muck lands develop the disease when grown on relatively raw peat soils (willow and elder type) high in available nitrogen and low in total mineral content, especially silica.

The absence of leaf rust (*Puccinia purpurea*) among the hybrid progeny is also of special interest, since all of the locally grown varieties of *Holcus sorghum* var. *saccharatus* readily become infected with this fungus.—B. A. BOURNE, Florida Agricultural Experiment Station, Gainesville, Florida.

*Felicia amelloides* Voss, *an Unreported Host for Erysiphe cichoracearum* DC.—The blue Agathea daisy, popular both as an outdoor garden flower and for potting or winter cutting in greenhouses, has been found attacked rather severely by *Erysiphe cichoracearum* DC. in Rhode Island. No report of a powdery mildew on this host, *Felicia* (*Agathea*) *amelloides* Voss, could be found, although the fungus has been reported on various other members of the Compositae.

A group of diseased plants growing in a bench in the college greenhouses was called to the writer's attention in the past winter. The lower leaves, especially, were covered with the white mycelium and conidiophores of the mildew. Also many scattered patches of dark reddish brown perithecia were present. Infected leaves became dry, died prematurely, and dropped off. The perithecia are typical of *Erysiphe cichoracearum;* each contains 8 or more asci and each more or less stalked ascus usually contains 2 ascospores. The haustoria are simple. A limited amount of material is available for distribution to herbaria.—FRANK L. HOWARD, Rhode Island State College, Kingston, Rhode Island.

## BOOK REVIEW

Arthur, Joseph Charles. *Manual of the Rusts in United States and Canada.* ii–xv + 438 pp. 487 figs. Illustrations by George B. Cummins. Purdue Research Foundation, Lafayette, Indiana. 1934.

Unfortunately, the general mycologist and plant pathologist has found it difficult to utilize the monograph of the rusts of North America published in the North American Flora (N. Am. Flora **7**: 85–969). This is partly due to use of life cycles in the separation of genera, with the resulting employment of unfamiliar names for the most common plant pathogens, names that have found little or no use in the literature of plant pathology and mycology. It is partly due to the difficulty of using a monograph published in parts over a period of 20 years with the resulting changes in concepts, classification, and terminology that naturally occur with accumulating knowledge. It therefore is fortunate that Dr. Arthur has incorporated the results of his years of intensive study of the rusts in a compact manual in the preparation of which one of the main objects has been "to produce a work to be serviceable in the determination of collections of rusts by the general botanist."

In the *Manual of the Rusts in United States and Canada,* life cycles have not been employed in the separation of genera. As a result, the classification is much simpler and easier to use and, what is probably of more importance to the general pathologist, the names employed are for the most part those in general usage. Also, in so doing, another object of the manual, the presentation of a classification showing relationship of species and genera, has been furthered.

The preface deserves careful study, since in it several innovations are discussed that should be clearly understood if the manual is to be utilized without confusion. Considerable emphasis is placed on the relationship of the various spore forms to the gametophytic (haploid) and sporophytic (diploid) states. Pycnia and aecia are considered the products of the gametophyte and uredia and telia of the sporophyte. As the term is employed in the manual, an aecium is the diploid spore form produced by the gametophytic mycelium of a macrocyclic rust. On this basis the sori containing pedicellate spores accompanying the pycnia of a brachypuccinia (primary uredinia of the N. Am. Flora) are uredinoid aecia, while the similar sori resulting from them (secondary uredinia of the N. Am. Flora) are uredia. Likewise, in some opsis rusts, infection from aeciospores may produce aecidia which in the terms of the manual would be aecidioid uredia (secondary aecia of the N. Am. Flora). Since the terms aecium, etc., are not based upon morphology, which is indicated by the modifying adjectives, it is unfortunate that such adjectives are used in only a few species. Apparently, in all cases, uredinoid aecia are so designated. However, in most of the species only the unmodified term aecium is employed. It is left for one to assume that these have catenulate aeciospores, produced in

aecidioid aecia. The terms uredia and urediospores are used to replace uredinia and urediniospores (Ber. Deutsch. Bot. Gesellsch. 50a: 25. 1932). It is somewhat questionable whether any important advantage is derived.

On the basis of life cycle the rusts are divided into macrocyclic, having all spore forms or with uredia lacking (demicyclic) and microcyclic, having telia with or without pycnia. The latter are considered reduced forms.

In general, the International Code of Nomenclature has been followed with the exception that "the date for beginning of nomenclatural priority is taken to be 1753, not 1801." It is stated that this change affects only 5 specific names, resulting in the retention of names in common usage. A discussion of the phase "perfect state" of the International Code is given, in which it is maintained that this should be interpreted to apply to the sporophytic state and that, therefore, Uredo names are permissible. Such an interpretaton, of course, would exclude Uredo names applied to uredinoid aecia.

Two families are recognized, the Melampsoraceae, which is divided into 4 tribes—Pucciniastreae, Cronartieae, Melampsoreae and Phakopsoreae—and the Pucciniaceae with 3 tribes—Ravenelieae, Phragmideae, and Aecidieae. The Melampsoraceae are characterized by sessile teliospores, the Pucciniaceae by pedicellate teliospores.

The genera Uredinopsis, Milesia, Hyalopsora, Pucciniastrum, Melampsorella, Melampsoridium, Cronartium, Chrysomyxa, Coleosporium, Melampsora, Aplopsora, Bubakia, Physopella, Cerotelium, and Baeodromus are treated in the Melampsoraceae. For the genus Milesia, Faull's recent monograph is followed. Thecopsora and Calyptospora are treated as sections of the genus Pucciniastrum.

The genera Ravenelia, Pileolaria, Tranzschelia, Cumminsiella, Uropyxis, Phragmopyxis, Phragmidium, Xenodochus, Frommea, Kuehneola, Mainsia, Gymnoconia, Trachyspora, Triphragmidium, Nyssopsora, Puccinia, Uromyces, and Gymnosporangium are discussed in the Pucciniaceae. Species of Uromyces are not treated as a separate group, but are distributed with species of Puccinia according to their relationship. The genus Puccinia is divided into 2 sections, Eupuccinia and Bullaria. The statement concerning the separation of the 2 sections is somewhat misleading. It is stated (p. 111) that the aecia of the species belonging to the section Bullaria are uredinoid with echinulate aeciospores, less often aecidioid with verrucose aeciospores. This statement, which would hold for the genus Bullaria as used in the North American Flora, does not agree with the data given for the species placed in the section Bullaria, since most of these have aecidioid aecia and verrucose aeciospores. The separation, which seems to be a logical one, is determined principally by the shape and character of the wall of the teliospores. Under the heading of Form-genera, unconnected stages are placed in Aecidium, Peridermium, Caeoma, Uraecium (uredinoid aecia), and Uredo.

Keys to the genera are given under each family and to the species under each genus. In the keys to species, correlated species, although retaining their specific identity, are listed, following the most fully developed species. Only the latter is given a number. Demicyclic and microcyclic species are indicated by the designations (d) and (m), respectively. Species of Uromyces and Endophyllum are similarly placed. By this means, closely related species are grouped together. Each numbered group of species "may in a way be considered to represent an ancestral species broken into more or less distinct forms, now called correlated species, during evolutionary development." This interesting innovation has the advantage of emphasizing the close relationship of groups of species that, in most treatments of the rusts, are widely separated when arranged on the basis of hosts or are placed in separate genera because of differences in life cycle or morphology.

The specific descriptions are concise, but include important diagnostic characters. Only the most important synonyms are listed. References, however, are given to the North American Flora where more detailed information is available. The descriptions are accompanied by 487 excellent text figures by George B. Cummins. These greatly aid in visualizing the species and will be an important help in identification. A very valuable addition to the information concerning the species is a statement giving the results of cultures. This enables one to evaluate the assignment of hosts for the various stages and probably will stimulate studies in this field.

As stated in the preface, principal emphasis is placed upon morphologic differences in the separation of species. This has resulted in placing in synonymy a number of species that have been separated largely on the basis of host specialization. Most important in connection with the literature of plant pathology is the inclusion of *Puccinia triticina* and *Puccinia dispersa* as varieties in the species *Puccinia rubigo-vera*.

In a number of species, varieties are distinguished, part on a morphologic basis, part by host specialization, and part by geographic distribution. In a few cases it is somewhat questionable whether the distinctions made are really significant. Thus, *Puccinia rubigo-vera agropyri* and *P. rubigo-vera agroyprina* are apparently separated principally on the basis of range. They include groups, that can be distinguished much better by morphology and host specialization. Groups thus separated would conform more closely to the other varieties that are recognized in the species. No attempt is made to include the results of the extensive studies of physiologic specialization in the cereal rusts.

The Manual will unquestionably be very useful, not only to the general botanist but to the beginning student and the specialist, as well. It not only will serve as a very convenient and useful reference but also will clarify the taxonomic relationships of North American species.—E. B. MAINS, University of Michigan, Ann Arbor, Michigan.